A New Route to Oligonucleotides

by

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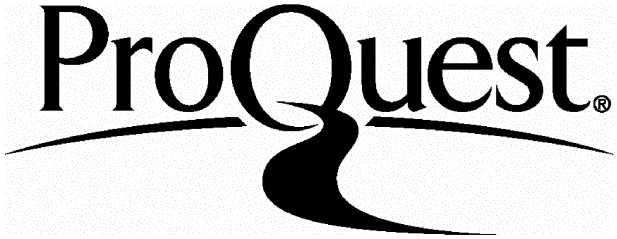
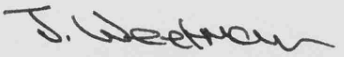


STATEMENT

The experimental work in this thesis, submitted for the degree of Doctor of Philosophy, entitled "A New Route to Oligonucleotides" has been carried out by the author in the Department of Chemistry at the University of Leicester between October 1982 and September 1985. All the work in this thesis is original, unless otherwise acknowledged in the text or by references. This work has not been, and is not currently being, presented for any other degree in this or any other university.



University of Leicester

November 1985

to Mum, Dad,

Mary Veronica

and Mick

Acknowledgements

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I am grateful to my fellow research workers, in particular Sally Freeman, John Davies and Paul Kay, the academic staff, in particular Dr Paul Cullis, for many helpful discussions and the technicians, especially Mick Lee, in the Department of Chemistry for their help and friendship.

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JOHN WEETMAN

ABSTRACT

A New Route to Oligonucleotides

The application of the Arbusov reaction to generate a nucleoside-containing phosphoryl halide for use in oligonucleotide synthesis has been investigated. In Arbusov reactions on mixed trialkyl phosphites, t-butyl halide is always lost in preference to methyl, ethyl or cyclopentyl halide. Exploitation of this apparent SN1 nature of the second stage of the reaction was proposed. The phosphitylating agent t-butyl methyl phosphorochloridite was found not to be isolable and t-butyl methyl N,N-dimethylphosphoramidite upon phosphitylation of a 5'-protected monomer using tetrazole as acid catalyst was prone to loss of isobutylene. The use of alternative groupings, namely, 2-methoxyethyl, 1-methoxypropan-2-yl, allyl, 1-methoxy-2-methylpropan-2-yl and 1-methylcyclopentyl to exploit the SN1 nature of the reaction proved unsuccessful.

Dimethyl phosphorochloridite was found to phosphitylate a 5'-protected monomer efficiently. Reaction with bromine proceeded with exclusive loss of methyl bromide. SN2 attack at methyl is thought to occur preferentially since bromide ion is sterically prevented from attacking the 3'-position by the nucleobase and the bulky trityl protecting group. The resulting phosphorobromidates were found to phosphorylate the 5'-hydroxyl of a 3'-protected monomer efficiently in solution in the presence of 1-methylimidazole. DMF, DMPU, pyridine and tetrazole have also been investigated as activating agents.

Using o-chlorophenyl as the internucleotide protecting group this procedure was unsuccessful but this led to a series of interesting acid catalysed ligand exchange reactions on aryl diethyl phosphites containing various substituted aryl groups, those with the most electron-withdrawing substituents generally exchanging most rapidly.

The route beginning from dimethyl phosphorochloridite was investigated on a CPG polymer support. A thymidine residue bearing an acid labile 5'-O-protecting group and attached through its 3'-position via a succinate linkage and a LCAA spacer to the polymer support was deprotected with trichloroacetic acid. Subsequent phosphorylation of the free 5'-hydroxyl was found to be inefficient, the reason suggested being that the longer phosphorylation reaction times required on the solid support allowed demethylation of the active phosphorylating agent by halide ion to occur.

Contents

Experimental

List of Abbreviations and Symbols

Ar	Aryl
Ph	Phenyl
Et	Ethyl
Me	Methyl
Bu^t	t-butyl
DMTr	Dimethoxytrityl
MMTr	Monomethoxytrityl
Tr	Trityl
Ac	Acetyl
Bz	Benzyl
ib	isobutyryl
an	p-anisoyl
THF	tetrahydrofuran
DMF	N,N-dimethylformamide
DMAP	N,N-dimethylaminopyridine
DMPU	N,N'-dimethyl-N,N'-propylene urea
TCA	trichloroacetic acid
AcOH	acetic acid
DCCI	dicyclohexylcarbodiimide
DIPEA	diisopropylethylamine
T	Thymidine/Thymine
A	Adenine
C	Cytosine
G	Guanine
(TpT)	thymidylyl (3'→5')-thymidine
MSNT	1-mesitylenesulphonyl-3-nitro-1,2,4-triazole

CPG	Controlled pore glass
LCAA	long chain alkylamine
δ	chemical shift measured in parts per million (p.p.m.).
Δ	denotes reaction proceeds under thermal conditions.
^{31}P n.m.r.	phosphorus nuclear magnetic resonance spectroscopy.
^{1}H n.m.r.	proton nuclear magnetic resonance spectroscopy.
M^{+}	molecular ion
m/e	mass divided by charge
r.t.	retention time
R_f	retention factor
tlc	thin layer chromatography
Hz	Hertz
MHz	Mega hertz
KHz	Kilo hertz
mm/Hg	measurement of pressure in millimetres of mercury.
M	molar
i.d.	internal diameter
^{o}C	degrees centigrade
HPLC/hplc	high performance liquid chromatography
SAX	strong anion exchanger
s	singlet
d	doublet
t	triplet
m	multiplet
(b)	broad
q	quartet
mbar	millibar
i.r.	infra red
u.v.	ultra violet
cms	centimetres

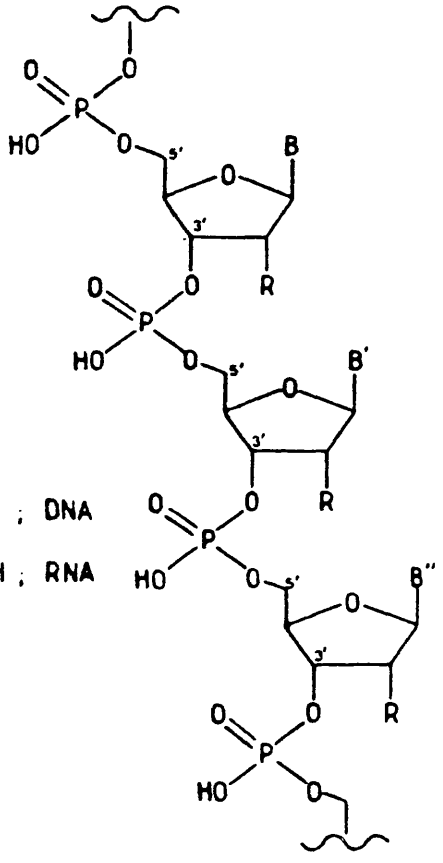
cm^3	cubic centimetres
w.r.t.	with respect to

CHAPTER 1

INTRODUCTION AND REVIEW OF CURRENT SYNTHETIC METHODOLOGIES

Introduction

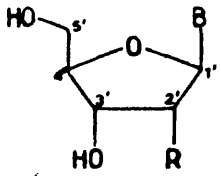
Oligonucleotides (1), structurally, are polydialkyl phosphate esters in which the 3'-hydroxyl group of one nucleoside unit (2) is joined to the 5'-hydroxyl group of another through a phosphodiester linkage.



(1)

(1a), R = H ; DNA

(1b), R = OH ; RNA

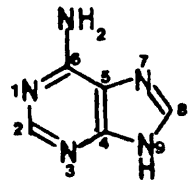


(2)

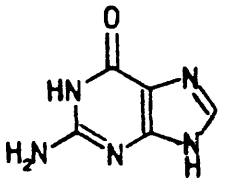
(2a), R = H

(2b) R = OH

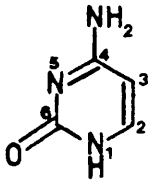
DNA and RNA differ in several respects, the most obvious of which is that in DNA, (1a), the nucleoside units are β-D-2'-deoxyribofuranosides of pyrimidine and purine bases, (2a), while in RNA, (1b), they are β-D-ribofuranosides of pyrimidine and purine bases. The four heterocyclic bases are attached to the primary structure at each 1' sugar position. RNA and DNA both contain the purine bases adenine and guanine, attached through their positions 9. Of the pyrimidines, both contain cytosine but in DNA the fourth base is thymine, whereas in RNA it is normally uracil. The pyrimidines are attached through their positions 1, see Figure 1.1. Chemically synthesised oligonucleotides of known base sequence have been of considerable value in biological studies. It is now possible to construct



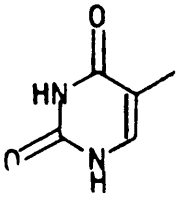
Adenine



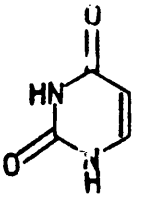
Guanine



Cytosine



Thymine



Uracil

Figure 1.1

DNA duplexes up to several hundred base pairs by enzymatically linking together short, chemically synthesised oligonucleotides, the enzyme being T4 DNA ligase. For example, Itakura[1] synthesised a gene for the peptide hormone somatostatin, using a strategy based on that of Khorana, which involved the ligation of eight overlapping chains 11-15 residues in length. Short oligonucleotides (~ 11 residues in length) have been used as primers for the sequencing of long strands of DNA, in particular, by the "dideoxy" chain termination method.[2] In site directed mutagenesis a primer is constructed with a desired mismatch from the template and after expression of the resulting double stranded DNA in a suitable host, mutant proteins can be generated containing specific amino acid changes. Short chemically synthesised DNA duplexes have also been used in X-ray crystal analysis[3] and two dimensional proton n.m.r. has provided a new technique for the study of DNA - protein recognition.[4] Therefore, there is considerable demand for chemically synthesised oligonucleotides and there is good reason to believe that these molecules will continue in the future to find important

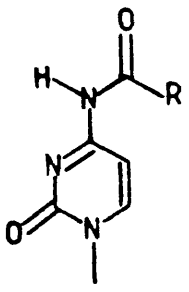
application in biological research.

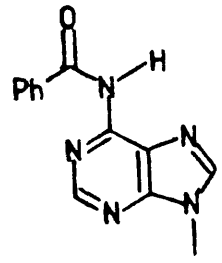
Synthetic Strategy

The key step in the synthesis of oligonucleotides is the formation of the internucleotide phosphate linkages. However, as a nucleoside unit (2) contains two hydroxyl groups (three in the case of an RNA monomer) at the 3' and 5' positions one must be selectively protected while the other is specifically phosphorylated (or phosphitylated) and then coupled to the next nucleoside unit. To date, more success has been achieved in the synthesis of oligodeoxyribo - than of oligoribonucleotides, this being due to the obvious necessity to protect the 2'-hydroxyl functions throughout the synthesis and then to expose them under conditions mild enough such that no side reactions occur. In addition, other reactive moieties, such as the exocyclic amino groups in adenine, cytosine and guanine must also be protected. Therefore, from the earliest days synthetic strategies have involved two kinds of protecting group. Firstly, a permanent protecting group which remains intact throughout the synthesis and then is removed at the end and secondly, a temporary protecting group which is present such that specificity can be obtained for a particular reaction and then removed immediately.

Protecting Groups

The amino functions of adenine, cytosine and guanine are susceptible to attack by electrophiles such as phosphorylating agents and therefore must be protected to prevent side reactions which otherwise would be observed at least to some extent during condensation reactions. Although alternatives have been proposed[5] most synthetic work has followed the initiative set by Khorana [6a,b] in that the protection of the amino functions is effected by acylation, Figure 1.2



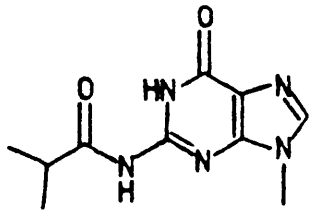


(4)

N^6- Benzoyl - 2'- deoxyadenosine (A^{bz})

(3a) R = Ph, N^4- Benzoyl-2'-deoxycytidine (C^{bz})

(3b) R = 4-MeO-C_6H_4, N^4-p-Anisoyl-2'-deoxycytidine (C^{an})



(5) N^2- Isobutyryl - 2'- deoxyguanosine (G^{ib})

Figure 1.2

The most popular protecting groups are benzoyl or p-anisoyl for the protection of cytosine, (3a) and (3b), benzoyl for adenine (4) and isobutyryl for guanine (5). Thymine is usually used without protection. The N-acyl groups are relatively stable in neutral and acidic media and are moderately stable at high pH. Thus, they may be retained while both acid- and base-labile protecting groups are removed from an oligonucleotide. The N-acyl groups themselves may be removed by ammonolysis.[7] The selective introduction of the N-acyl groups is achieved by excess acylation followed by selective removal exemplified in Figure 1.3 for adenosine.

Thus, treatment of 2'-deoxyadenosine (6) with an excess of benzoyl chloride in pyridine solution effects acylation of the 5'- and 3'-hydroxyl groups in addition to the required acylation of the N^6 position. The

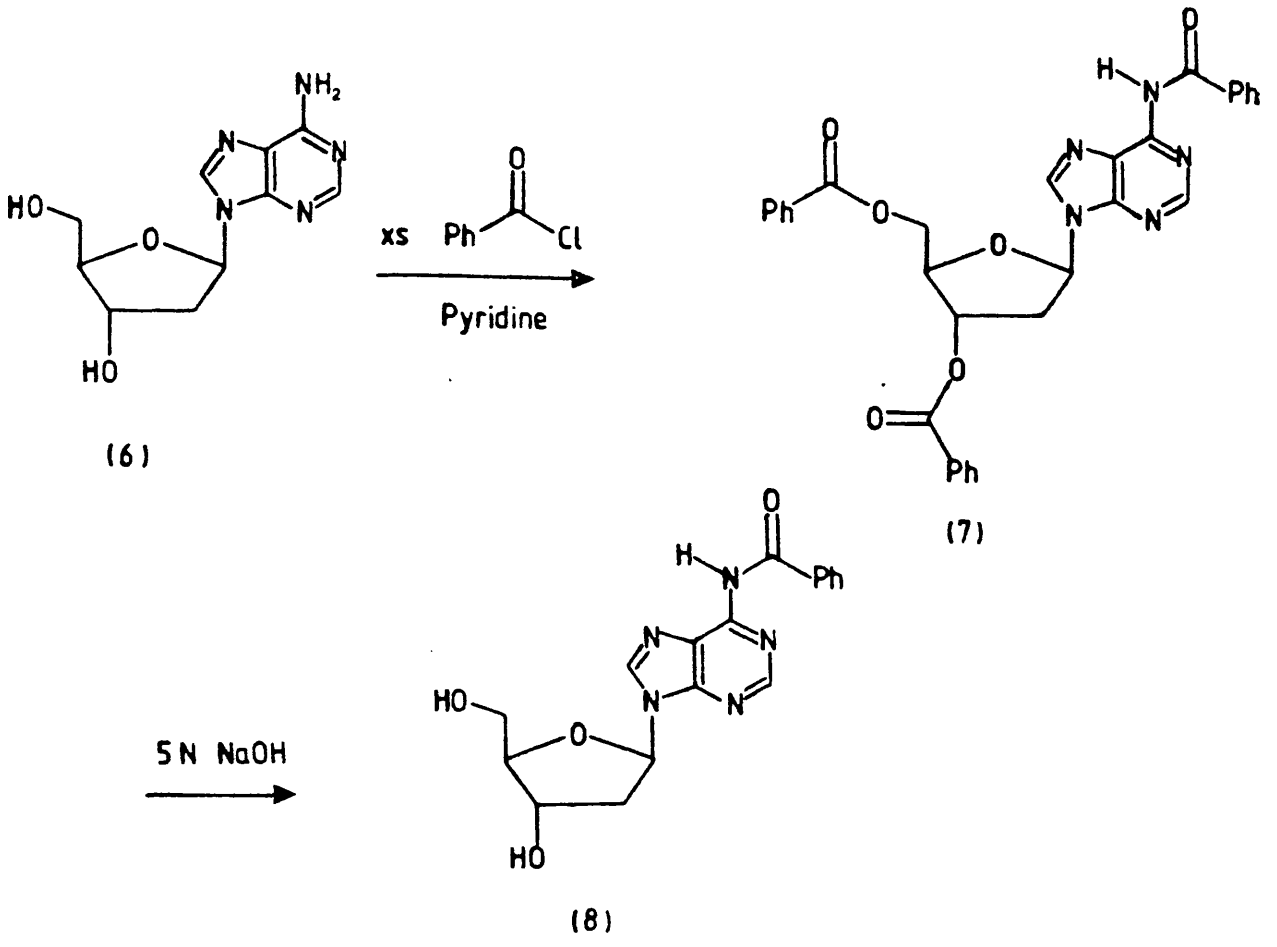


Figure 1.3

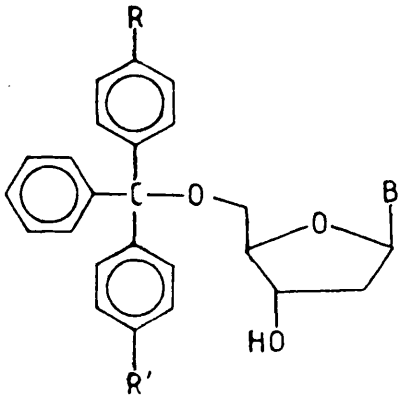
triacylated species (7) is not isolated but treated with sodium hydroxide to hydrolyse the esters but leave the amide intact to give N^6-benzoyl-2'-deoxyadenosine (8) which is then subjected to selective 5'-hydroxyl protection prior to phosphorylation (or phosphitylation).

In addition to the need for amino protection in the base residues, the identification of side reactions have, more recently, been observed in association with guanine[8] (in DNA synthesis)and uracil[9] (in RNA synthesis). This has prompted the introduction of a second protecting group at the O-6 position in guanine and the O-4 position in uracil.

Various research groups have approached this problem in different ways. Reese[10] has introduced the 6-O-(2-nitrophenyl) group and various 6-O- substituted silyl, sulphonyl, phosphoryl and phosphino-thioyl derivatives have been introduced by Hata.[11] Jones has introduced appropriately substituted ethyl groups prone to β-elimination[12] and Pfleiderer has investigated the use of the p-nitrophenylethyl group.[13] With regard to RNA

synthesis Reese has examined the possibility of using the phenyl group and the 2,4,dimethylphenyl group[10] to protect the O-4 position in uracil. These protecting groups also need to be selectively introduced and removed at the end such that base modification does not occur during extended synthesis.

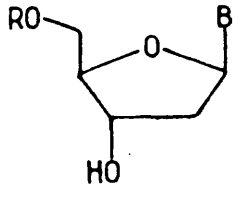
Protection at the sugar hydroxyls can be temporary or permanent depending upon synthesis strategy. It is common for the 2'-deoxyribonucleoside units to be phosphorylated (or phosphitylated) at the 3'- position, this therefore requiring a temporary protecting group at the 5'- position. This requirement is currently fulfilled by use of one of the acid-labile groups shown in Figure 1.4. The increasing lability to acid is in the order trityl < monomethoxytrityl < dimethoxytrityl. These groups were originally introduced by Khorana[15] and, because of their bulk, they functionalise selectively the primary 5'-hydroxyl position. An acceptable alternative to the trityl family is the 9-phenylxanthen-9-yl (pixyl)



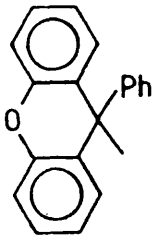
$R = R' = H$	Trityl, Tr
$R = H,\ R' = OCH_3$	Monomethoxytrityl, MMTr
$R = R' = OCH_3$	Dimethoxytrityl, DMTr

Figure 1.4

group (9), introduced by Reese[16]. The pixyl group has the advantage of conferring crystallinity on the protected nucleoside, a property the trityl



R =

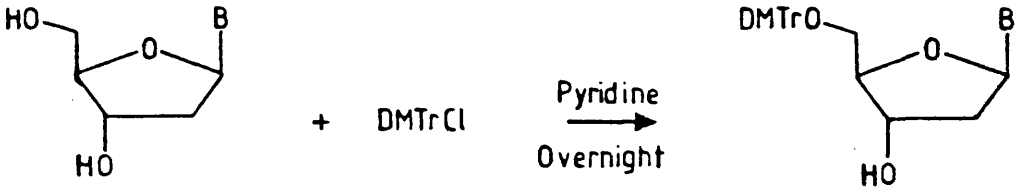


Pixyl

(9)

groups do not have. The dimethoxytrityl group and the pixyl group are extremely acid labile and need to be so since the glycosidic linkages of purine deoxyribonucleosides, in particular, N^6-benzoyl-2'-deoxyadenosine (8), are very sensitive to acid hydrolysis. Therefore, the more acid labile the protecting group, the more the problem of depurination is kept to a minimum.

The 5'-protected monomers are easily prepared by treatment of the 2'-deoxyribonucleoside (2a) with a slight excess of dimethoxytrityl chloride in pyridine solution and allowing the mixture to stir overnight to give the 5'-protected monomer (10), Figure 1.5. The dimethoxytrityl group is being



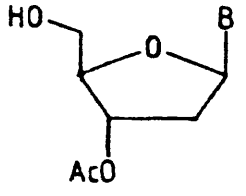
(2a)

(10a) B = T
(10b) B = C^{bz}
(10c) B = A^{bz}
(10d) B = G^{ib}

Figure 1.5

used here as a temporary protecting group and its removal can be effected by 10% TCA in methylene chloride or by 80% acetic acid.

In addition to the need for a 5'-protected building block, there is a requirement, if the synthesis is to be carried out by solution methods,[18] for a monomer protected at the 3'-hydroxyl position. The group generally used is the base labile acetyl group, as in (11a-d), and it was introduced



(11)

Ac = CH_3C(=O)

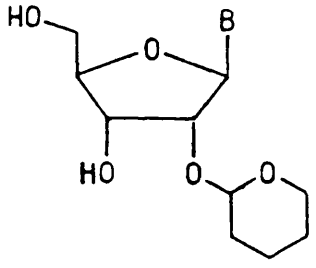
(11a) B = T
(11b) B = C^{bz}
(11c) B = A^{bz}
(11d) B = G^{ib}

by Khorana[7] to be used in conjunction with the 5'-protected monomers (10).

The 3'-protected monomers can easily be prepared in two steps from (10) by acylation, followed by acidic hydrolysis. Building blocks with 3'-acid-labile and 5'-base-labile protecting groups have also been prepared[19,20,21] although generally the 5'-hydroxyl is protected by an acid-labile group and the 3'-hydroxyl by a base-labile group.

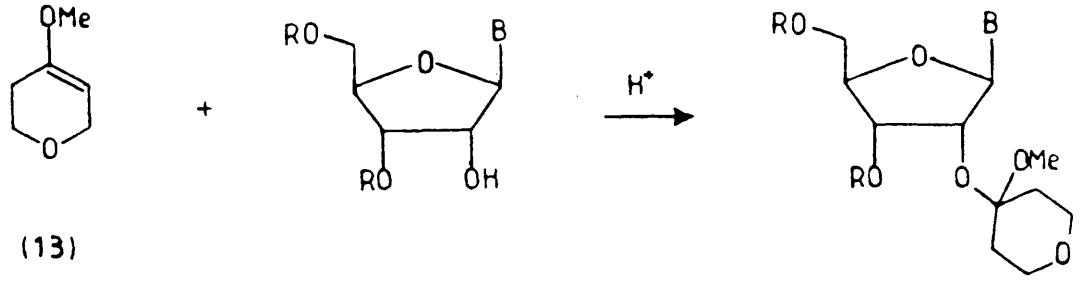
The synthesis of RNA fragments is more elaborate and time consuming due to the presence of the additional 2'-hydroxyl function. Therefore, in the synthesis of RNA fragments, three hydroxyls must be dealt with, an extra protecting group being required at the 2'-postion.

Initially, the tetrahydropyranyl acetal system was used,[22,7] as in (12), as it could be removed under mild enough acidic hydrolysis not to promote



(12)

excessive migration of the phosphoryl group to the 2'-position. However, the THP group is chiral and when used with optically active alcohols results in mixtures of diastereoisomers. However, acid catalysed addition of alcohols to 5,6,dihydro-4-methoxy-2H-pyran (13) gave compounds (14) which were found to have the desired hydrolysis properties. Thus, hydrolysis of (14) occurs in 24 minutes at 20 °C in 0.01M hydrochloric acid. Furthermore,



(13)

R = Acyl protecting group

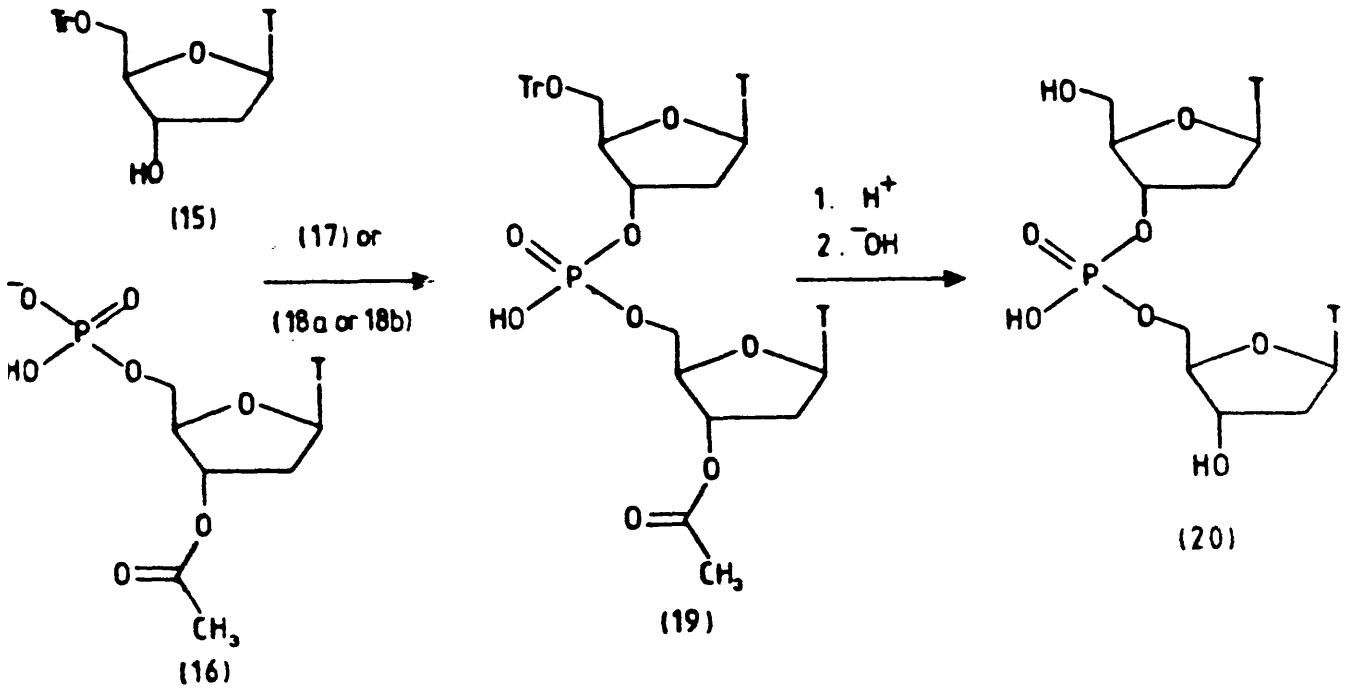
(14)

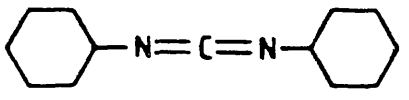
no diastereoisomers are formed as no new chiral centre is present in the protected derivative.

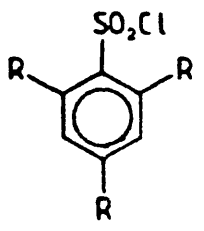
Internucleotide Coupling Procedures

The Phosphodiester Approach

The first effective approach to the synthesis of oligodeoxyribonucleotides is now commonly referred to as the phosphodiester approach. It was introduced by Khorana[23] and involves phosphorylation of intermediates with unprotected internucleotide linkages as shown in Figure 1.6.







(18a), R = Methyl

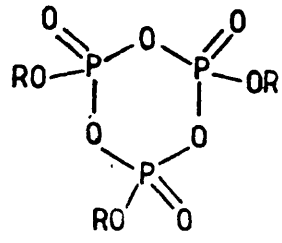
(18b), R = Isopropyl

Figure 1.6

A 5'-protected monomer (15) is allowed to react with a 3'-protected monomer phosphorylated at the 5'-postion (16) in the presence of N,N'-dicyclohexylcarbodiimide[24] (17), or an arenesulphonyl chloride (18), such as mesitylenesulphonyl chloride[25] (18a) or triisopropylbenzenesulphonyl chloride[26] (18b) in anhydrous pyridine solution. The dinucleoside phosphate obtained, (20), in 90-95% yield following deprotection is the simplest dimer initially to attempt to synthesise as thymidine is the easiest nucleoside to work with since it is usually used without base protection.

The mechanism of activation of the phosphate group is complex and the actual phosphorylating agent in the case of DCCI and the arenesulphonyl chlorides may be different[25] since the arenesulphonyl chlorides effect much more rapid phosphorylation.

It has been shown[27] that the reaction of a monoester with DCCI leads rapidly to the formation of a trimetaphosphate (21) and that this is probably



(21)

the initial phosphorylating species.

However, (21) is not a rapid phosphorylating agent and therefore must be subsequently activated, a process that would rely upon nucleophilic character from the phosphoryl oxygen. A possible mode of formation of (21) using an arylsulphonyl halide as activating agent is shown in Figure 1.7

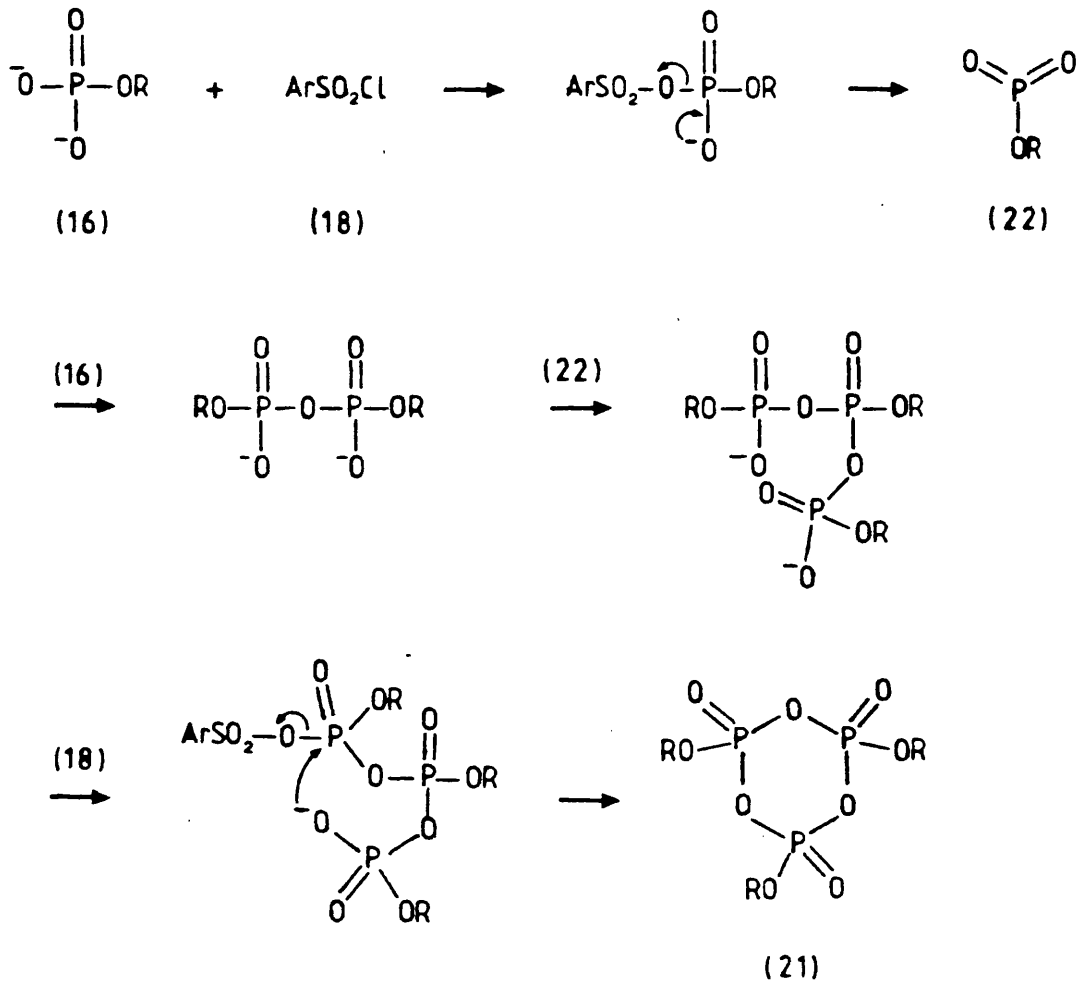
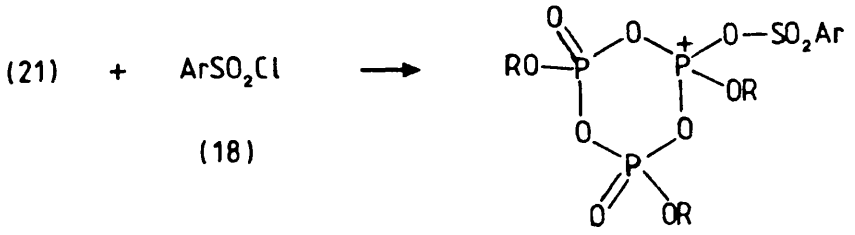
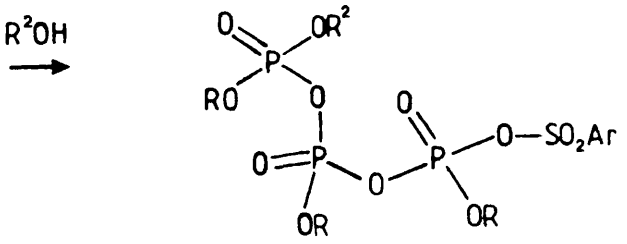


Figure 1.7

Subsequent activation of (21) must occur to render a neighbouring phosphate group open to nucleophilic attack from the second nucleoside component as speculated overleaf.



(18)



(23)

R^2OH = (15)

Compound (23) may undergo repeated anhydride exchange to result in the eventual required diester. Alternatively, direct combination of the monomeric metaphosphate (22), Figure 1.7, with the second nucleoside component (15) would give the diester directly. However, despite considerable achievements[6] utilising the phosphodiester approach it suffered certain limitations as exemplified by studies carried out by Griffin and Reese[28] in that:-

(i) Phosphodiester functions are nucleophilic and hence are potential sites of attack in subsequent phosphorylations thus competing with the 3'-hydroxyl.

(ii) Salts of phosphodiesters are soluble, normally, only in water or the more polar organic solvents. Therefore, normal purification techniques such as adsorption chromatography on alumina or silica gel cannot be used.

(iii) The technique of anion exchange chromatography involves the use of aqueous or aqueous alcoholic buffer solutions. Considerable care is required in the purification of partially protected phosphodiesters by this technique as the protecting groups employed are by necessity sensitive to acid - or base-catalysed hydrolysis.

(iv) Only moderate yields were obtained in the synthesis of long, high molecular weight, oligonucleotides by the phosphodiester approach as the phosphorylation reactions become less selective for longer chains.

The route is now of only historical and developmental interest.

The Phosphotriester Approach

The development of a protecting group for the internucleotide linkages became of great importance, as a consequence of the hazards of the phosphodiester route, and led to syntheses involving phosphotriester intermediates. The corresponding transformation (10) - (24) using the phosphotriester approach is shown in Figure 1.8.

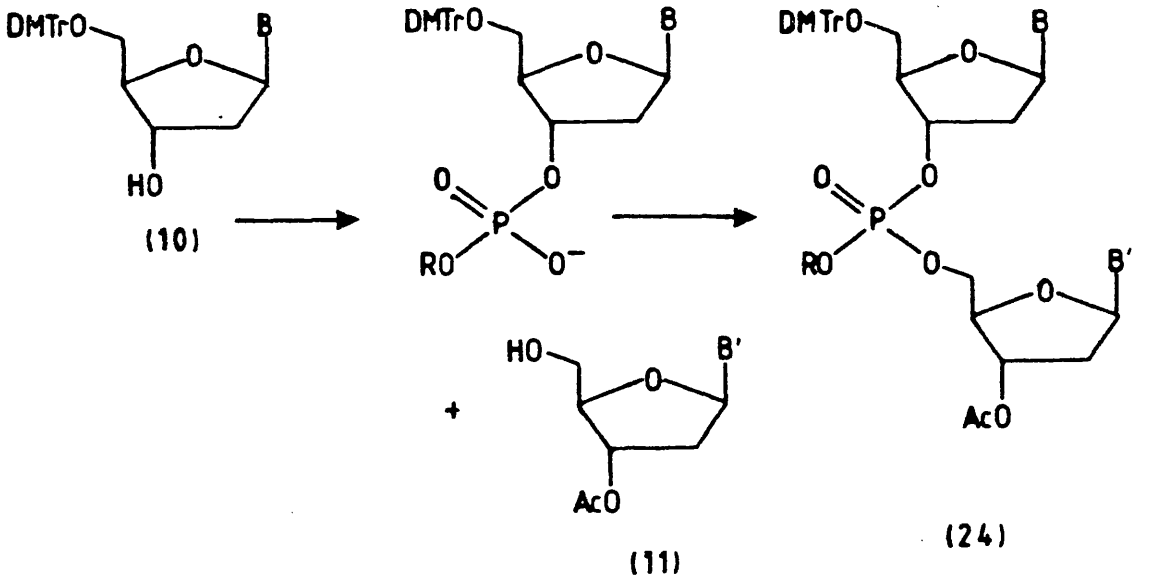


Figure 1.8

Clearly, the additional problem is the choice of protecting group R to block the internucleotide linkages. It must meet several requirements.

(i) It must be relatively easy to introduce

(ii) It must be stable under phosphorylation conditions and conditions to remove other protecting groups.

(iii) It must be removed at the end of the synthesis such that the required product is stable.

It was in 1955 that Michelson and Todd[29] first investigated the use of a protecting group for the internucleotide linkage, the benzyl group being their choice. Figure 1.9 shows their phosphotriester synthesis of thymidylyl (3'→5')-thymidine, TpT (20).

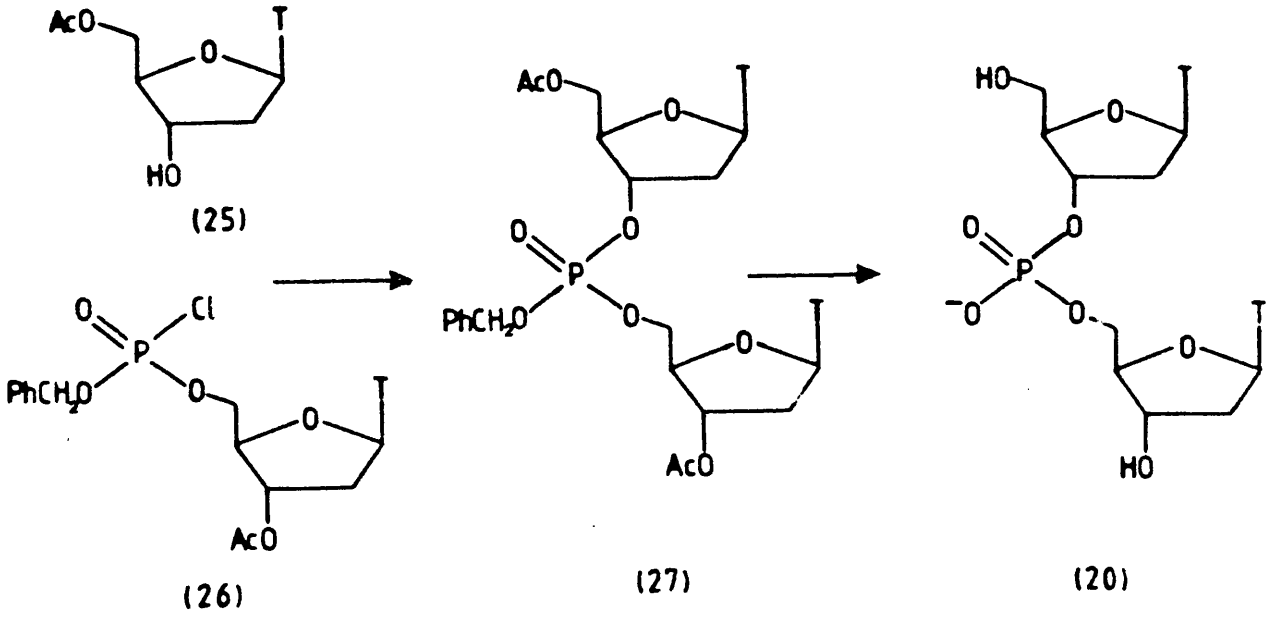


Figure 1.9

5'-O-Acetylthymidine (25) when treated with 3'-O-acetylthymidine-5'-O-(benzyl phosphorochloridate) (26) gave the fully protected dinucleotide (27). The benzyl group was removed by catalytic hydrogenation (H_2/Pd), although an alternative debenzylation procedure by treatment with lithium thiophenolate has, more recently been reported by Daub and Van Tamelen.[30] Base catalysed hydrolysis produced TpT (20).

Letsinger examined a similar approach using the β-cyanoethyl group to protect the internucleotide linkage.[31]

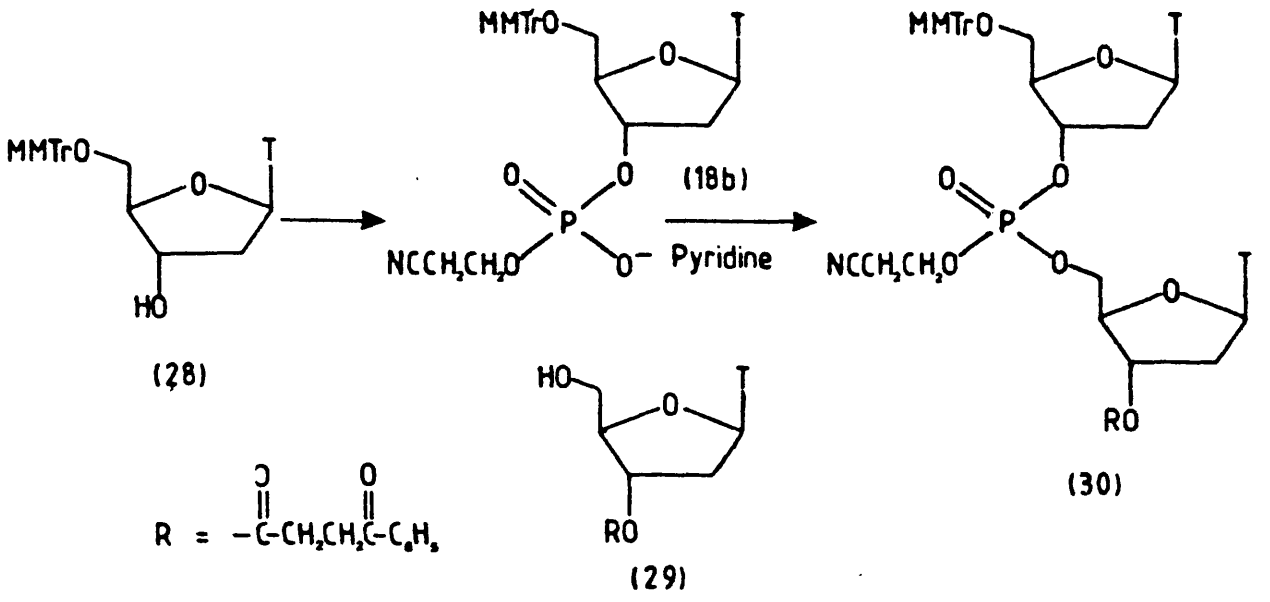


Figure 1.10

5'-O-Monomethoxytritylthymidine (28), Figure 1.10, was phosphorylated with β-cyanoethyl phosphate followed by reaction with 3'-O-β-benzoylpropionylthymidine to give the fully protected dinucleotide (30). Treatment with 80% acetic acid removed the monomethoxytrityl group and dilute ammonia removed the β-cyanoethyl group. Using this procedure, Letsinger was able to carry out the stepwise synthesis of oligodeoxyribonucleotides containing bases other than thymidine.

Eckstein and Rizk[32] demonstrated that the 2,2,2-trichloroethyl group

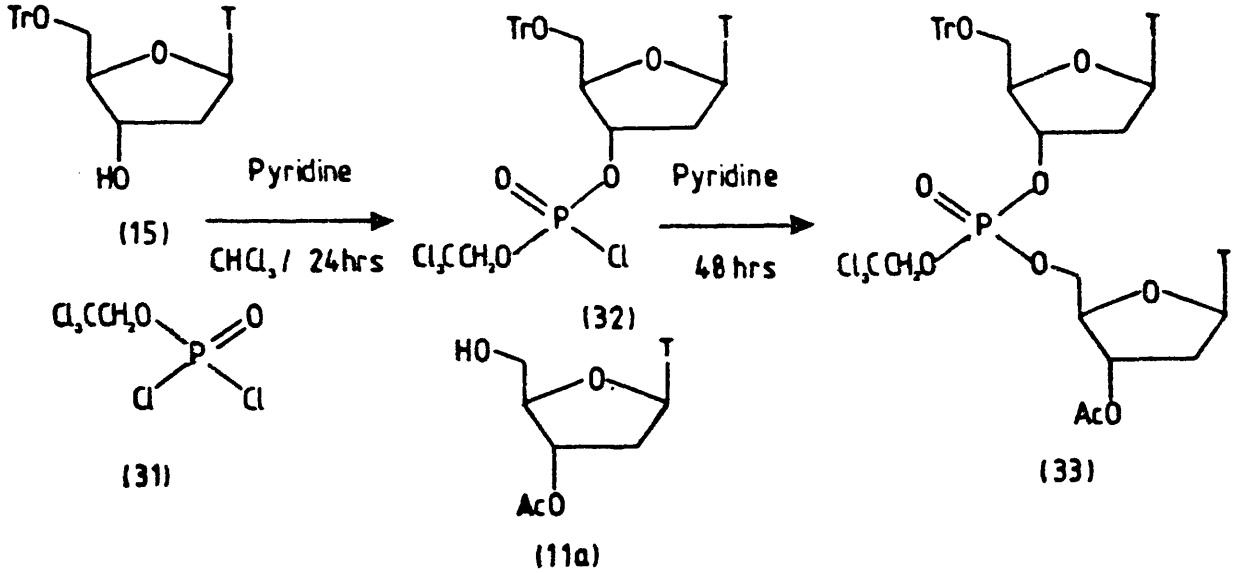


Figure 1.11

could be used to protect internucleotide linkages, Figure 1.11, in that treatment of 5'-O-tritylthymidine (15) with one molar equivalent of 2,2,2-trichloroethyl phosphorodichloridate (31) and pyridine, over a period of 24 hours gave 5'-O-tritylthymidine-3'-O-(2,2,2-trichloroethyl phosphorochloridate) (32). Subsequent treatment with 3'-O-acetylthymidine (11a) in pyridine for 48 hours resulted in, after chromatography, a 56% yield of the fully protected dinucleotide (33). The 2,2,2-trichloroethyl group was stable under the conditions to remove the hydroxyl protecting groups and was itself removed by treatment with zinc dust in 80% acetic acid at room temperature.

The attractive feature of the benzyl, β-cyanoethyl and 2,2,2-trichloroethyl groups was that their removal involved specific O-alkyl fission and did not involve nucleophilic attack at phosphorus. Thus, in the deblocking reactions the possibility of loss of the nucleoside residues was eliminated. However, the benzyl group is susceptible to nucleophilic attack, the β-cyanoethyl group is very sensitive to base and the treatment required to remove the 2,2,2-trichloroethyl group (Zn dust in 80% AcOH) has been shown to result in a decrease in the ultraviolet absorption, in particular in the case of N^4-benzoyl-2'-deoxycytidine,[33] suggesting some partial reduction of the pyrimidine ring. Furthermore, it should be emphasised that the successful synthesis of high molecular weight oligonucleotides has not[34] been reported using any of the three internucleotide protecting groups discussed.

Reese and Saffhill[35] showed that the phenyl group could be used to block the internucleotide linkages, demonstrating this in the synthesis of the fully protected TpT, (37), Figure 1.12. Thus, 5'-O-methoxytetrahydropyranylthymidine (34) was treated with phenyl phosphorodichloridate (35) and 2,6-lutidine in anhydrous acetonitrile solution. After 36 hours, conversion into the 5'-O-methoxytetrahydropyranylthymidine-3'-O-(phenyl

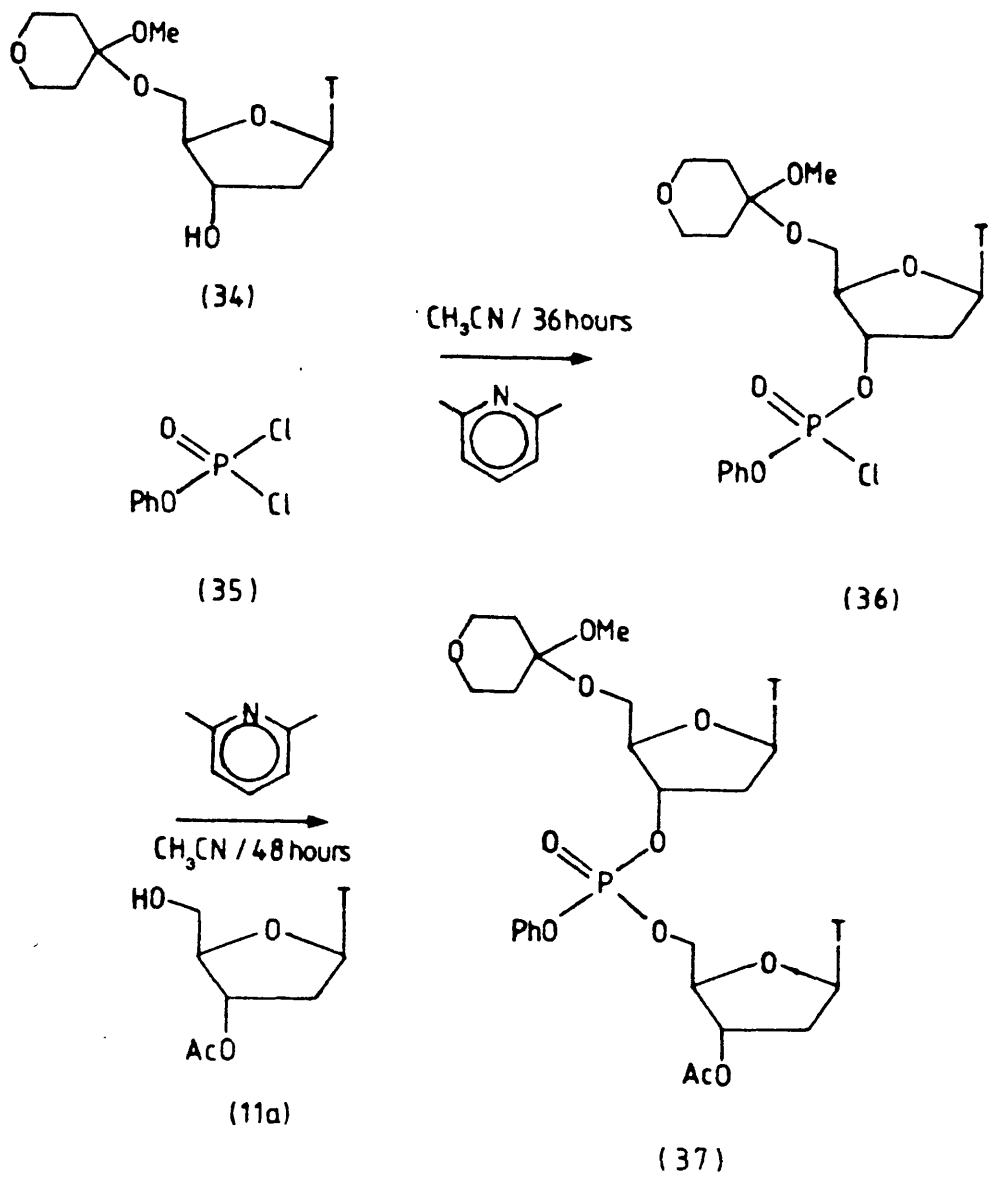


Figure 1.12

phosphorochloridate) (36) was achieved at which point 3'-O-acetylthymidine (11a) with an excess of 2,6-lutidine was added. After 48 hours the fully protected dinucleotide (37) was obtained in 70% yield.

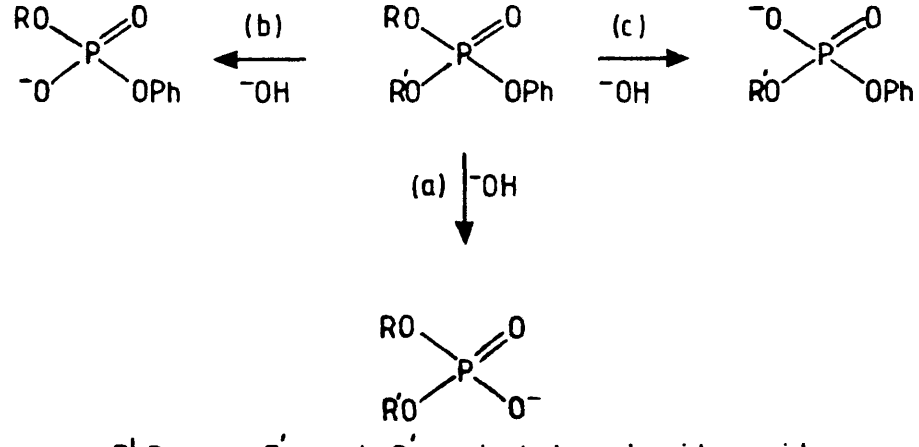


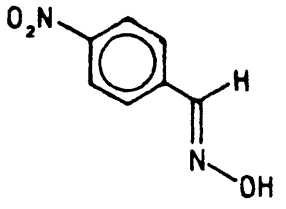
Figure 1.13

Experiments to remove the phenyl group by treatment with hydroxide ion, Figure 1.13, route (a), were initially carried out. Although this procedure involves nucleophilic attack at phosphorus and subsequent P-O fission, therefore rendering it disadvantageous compared to the benzyl, β-cyanoethyl and 2,2,2-trichloroethyl groups, it was envisaged that deprotection would occur virtually exclusively along pathway (a) as phenol is a stronger acid than simple alcohols by several orders of magnitude. This, however, is not the case and significant amounts of internucleotide cleavage (between 3 and 7%) have been observed resulting from pathways (b) and (c).[36 37]

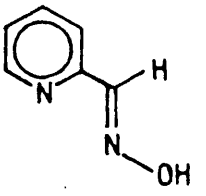
There are two logical approaches to consider in order to overcome this problem. The first is to use an aryl protecting group derived from phenol which is more acidic than phenol itself. However, for the internucleotide cleavage to be kept at, or below 0.5% per internucleotide link, the pK_a of the phenol must not be lower than 7.5[38] (e.g. o-NO_2, pK_a 7.2) because phenols of this acidity render the subsequently derived phosphotriesters extremely sensitive to alkaline hydrolysis and therefore they become difficult to handle.

The second approach is to use a nucleophile other than hydroxide ion in the deblocking step. Ammonia[39] in aqueous solution and tetraalkyl ammonium fluorides[40 41] have been used as alternative nucleophiles. However, ammonia can lead to phosphoramidate formation (~ 10%)[38] and internucleotide cleavage[42] as can fluoride ion.

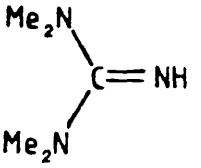
An acceptable compromise was developed by Reese[43] by using the o-chlorophenyl group (pK_a 8.5) to protect the internucleotide linkage and deprotect with the conjugate bases of syn-4-nitrobenzaldoxime (38) or syn-pyridine-2-aldoxime (39), together with the conjugate acid of N,N,N',N'-tetramethylguanidine (40).



(38)

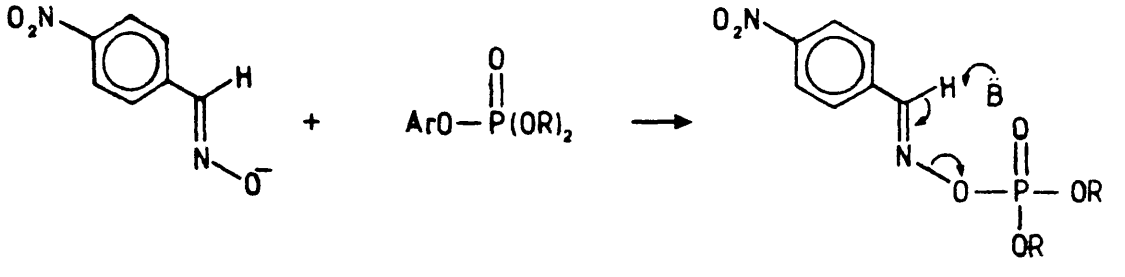


(39)



(40)

Deblocking is complete in under 3 hours at 20 ^{o}C when a ten fold excess (per phosphotriester group) of the conjugate base of (38) or (39) in aqueous dioxan solution is used and this results in only 0.5-1% detectable internucleotide cleavage. The mechanism of deprotection involves slow, rate determining nucleophilic attack at phosphorus to give the oximate ester (41), Figure 1.14, followed by rapid base catalysed elimination to give p-nitrobenzonitrile (42) and the dialkyl phosphate[44] (43)



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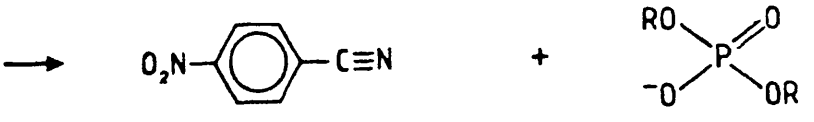


(42) (43)

Ar = o-chlorophenyl

R = Ethyl

Figure 1.14

Phosphorylation Steps

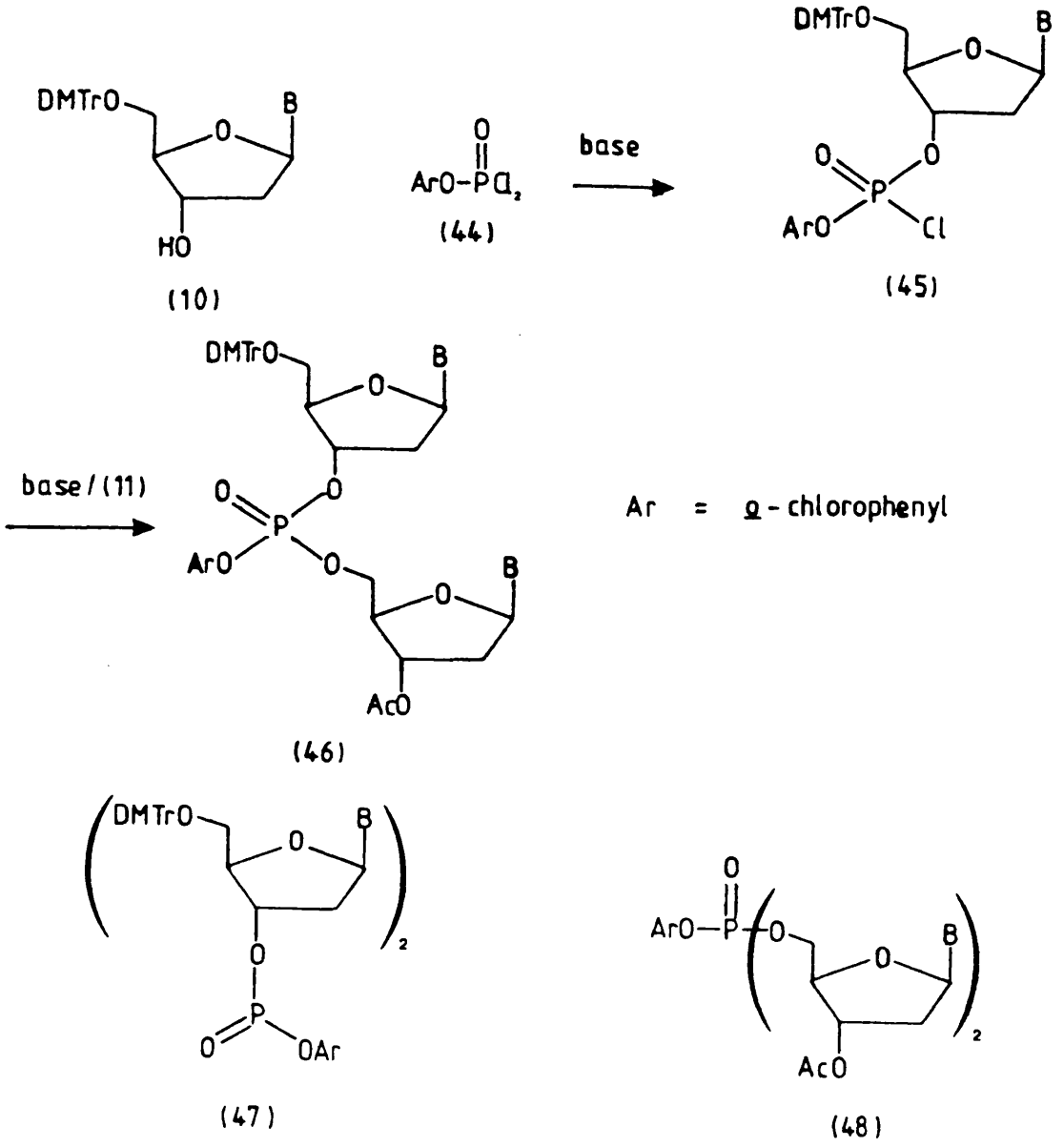


Figure 1.15

When a bifunctional phosphorylating agent is used, such as o-chlorophenyl phosphorodichloridate (44) in the phosphotriester approach, Figure 1.15, attempting to promote monosubstitution to give the phosphorochloridate (45) a significant degree of disubstitution is observed[45] to give the symmetrical (3'-3') dinucleoside phosphate (47). If stoichiometric quantities are used then the remaining phosphorodichloridate (44) can compete with (45) for the 3'-protected monomer (11). In this way, the symmetrical (5'-5) dinucleoside phosphate (48) is formed in addition to the

(3'-5')dinucleoside phosphate (46). Therefore, it is desirable that the phosphorylating agent be monofunctional.

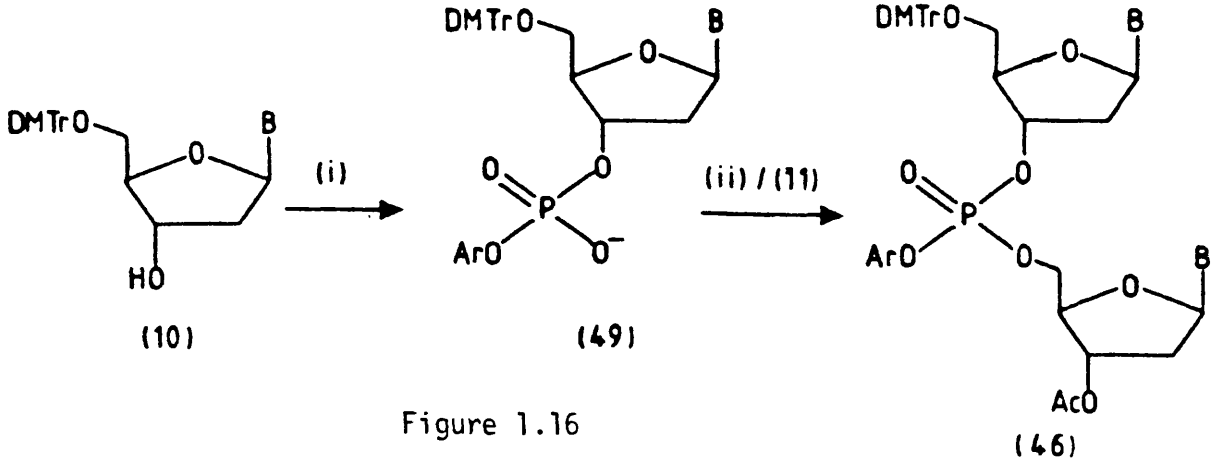
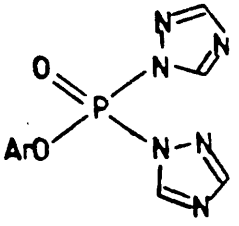


Figure 1.16

Figure 1.16 shows a modified version of Figure 1.15. The first phosphorylation step requires the conversion of (10) into the aryl nucleoside phosphate (49). After the use of aryl phosphorodichloridates had been discarded to carry out this transformation directly various research groups in recent years have examined a variety of monofunctional phosphorylating agents with which to carry out step (i).

The most commonly used methodology, however, is that developed by Reese and Chattopadhyaya[49]. They showed that o-chlorophenyl phosphorodi-(1,2,4-triazolide) (50) could be used to effect the required transform-



(50)

Ar = o-chlorophenyl

ation [step (i), Figure 1.16]. Although this reagent is bifunctional, and its analogue, with Ar = p-chlorophenyl has been used as such[39], it is effectively monofunctional when used in excess. Therefore, o-chlorophenyl phosphorodichloridate (44) when added to an excess of 1,2,4-triazole and

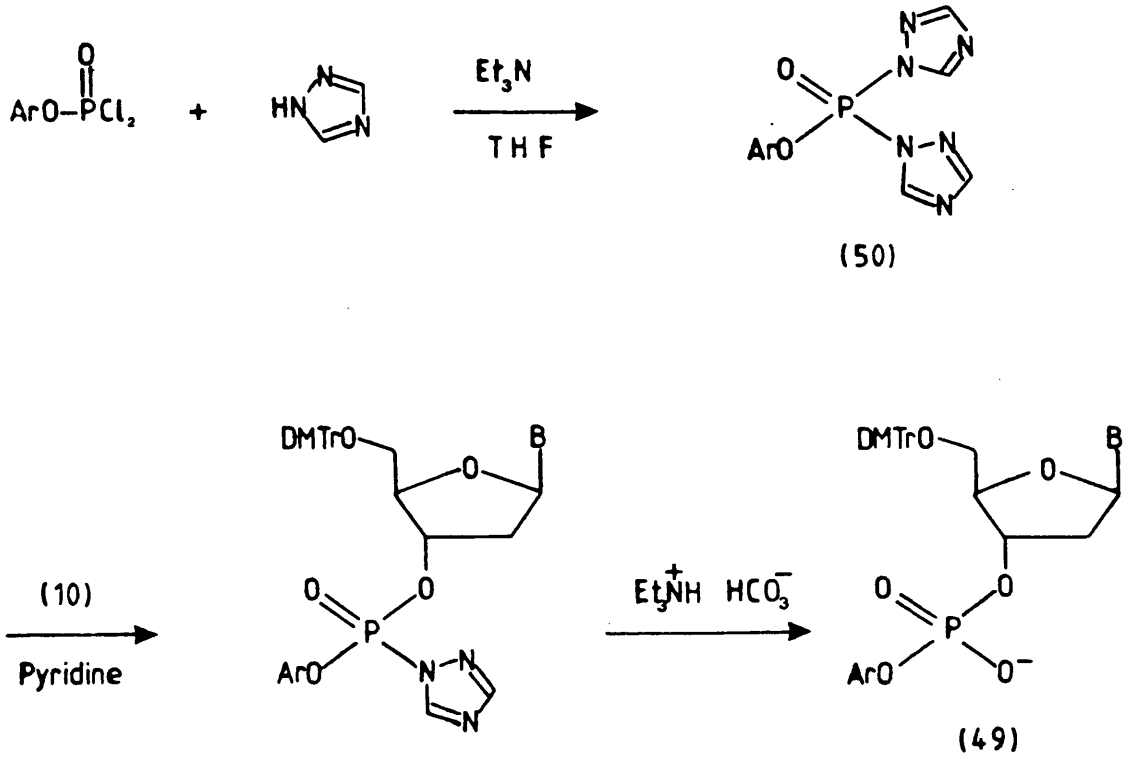


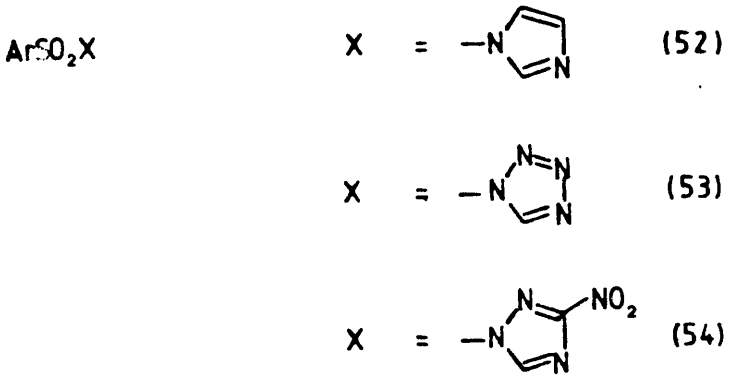
Figure 1.17

triethylamine in THF gives (50), Figure 1.17. This is then transfered (1.5 molar equivalents) to a solution of the 5'-protected monomer (10) in pyridine. After an hour, the monotriazolide phosphorylating agent is hydrolysed by addition of an aqueous solution of triethylammonium bicarbonate. Typical yields of (49) are 94%.

Having carried out the preparation of the phosphodiester (49), therefore completing steps (i), Figure 1.16, the second phosphorylation step, to form the fully protected phosphotriester (46), still requires the presence of an activating agent. Triisopropylbenzenesulphonyl chloride (18b), used by Khorana[26] as an activating agent in the phosphodiester approach can be used although the condensation reactions then tend to be rather slow. In addition, sulphonation of the 5'-hydroxyl function appears to compete with phosphorylation and darkening of the reaction mixture occurs. However, use of arenesulphonyl chlorides in the presence of the nucleophilic catalyst 1-methylimidazole (51) is reported[50] to effect relatively rapid phosphorylation (30 minutes) with only 2% sulphon-

(51)

ation of the 5'-hydroxyl function. In 1973, Russian workers reported[51] that the use of arenesulphonyl imidazole (52) did not lead to sulphonation

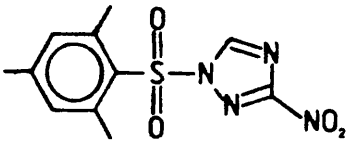


(52)

(53)

(54)

or darkening of the reaction mixture although the condensation reactions were rather slow. The same advantages are obtained when arenesulphonyl derivatives of tetrazole[52] (53) and 3-nitro,1,2,4-triazole[43] (54) are used and with these reagents the condensation reactions are relatively rapid and, indeed, the favoured condensation agent at present is 1-mesitylene sulphonyl-3-nitro-1,2,4-triazole[53], MSNT (55) and, in particular, MSNT in the presence



MSNT

(55)

of 1-methylimidazole (51).[54] The mechanism by which the second phosphorylation step proceeds has been studied by ^{31}P n.m.r. spectroscopy with[55] and without[56] the presence of the nucleophilic catalyst 1-methylimidazole. Both reports conclude that the fully protected phosphotriester is formed

in a two step process by initial formation of a (3'-3') symmetrical pyrophosphate tetraester (57), Figure 1.18.

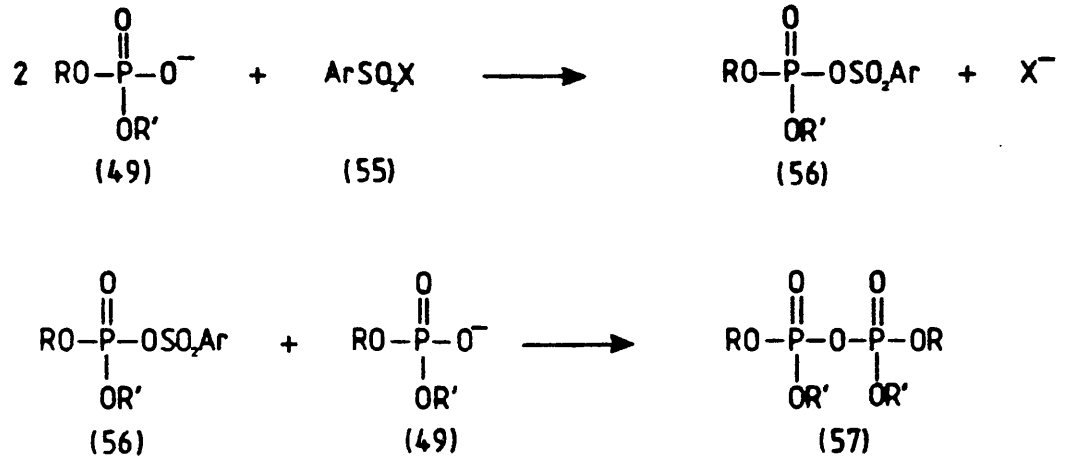
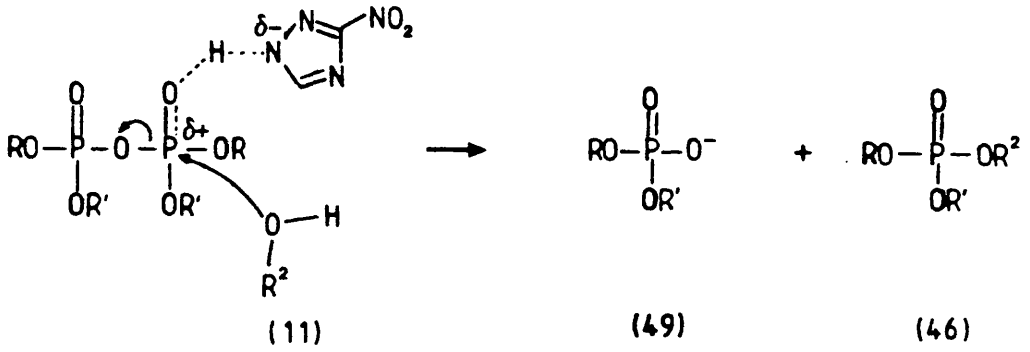


Figure 1.18

The phosphate diester (49) initially forms the mixed anhydride (56) by displacement of the 3-nitro-1,2,4-triazole form (55). Subsequent reaction of (49) with (56) forms the pyrophosphate (57). The route through to (57) is speculated[55] to involve 1-methylimidazole where this is present as part of the reaction mixture by initially attacking MSNT to give (58) which then reacts with (49) to give (56). The second step is the

(58)

condensation of the pyrophosphate (57) with the 3'-protected component (11) to give the required phosphotriester. The mechanism again differs depending upon whether 1-methylimidazole is present or not. With only MSNT present, acid catalysis is proposed[56] as the most likely mode of reaction, Figure 1.19.



(11) (49) (46)

R^2OH = 3'-protected monomer

R = o-chlorophenyl

R^1 = 5'-protected nucleoside residue

Figure 1.19

When 1-methylimidazole is present nucleophilic catalysis is proposed, Figure 1.20.

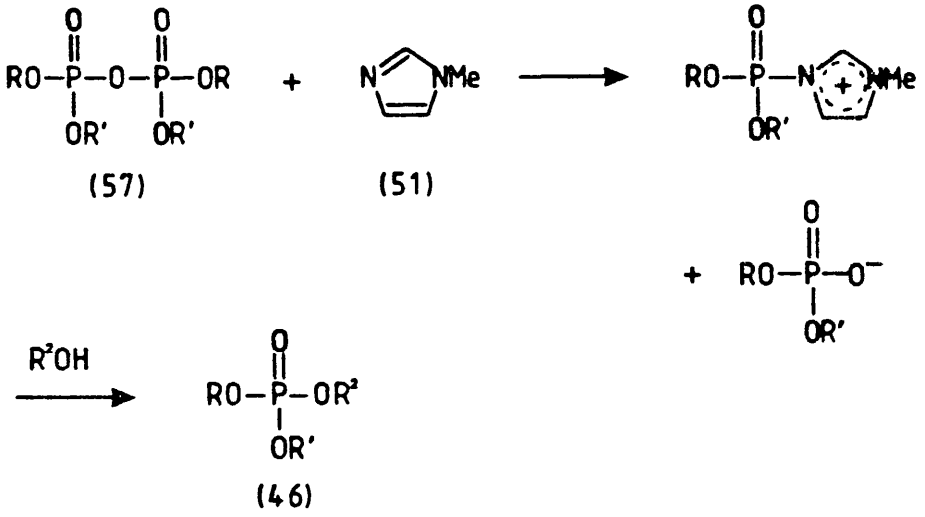
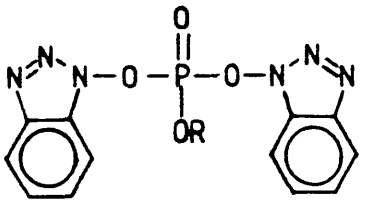


Figure 1.20

However, both reactions are carried out in pyridine solution and it has been suggested[57] that pyridine itself acts as a nucleophilic catalyst in an identical manner to that suggested for 1-methylimidazole. In the reaction where 1-methylimidazole is absent, pyridine may therefore fulfill a similar role suggesting the mechanism for each reaction may be

virtually identical.

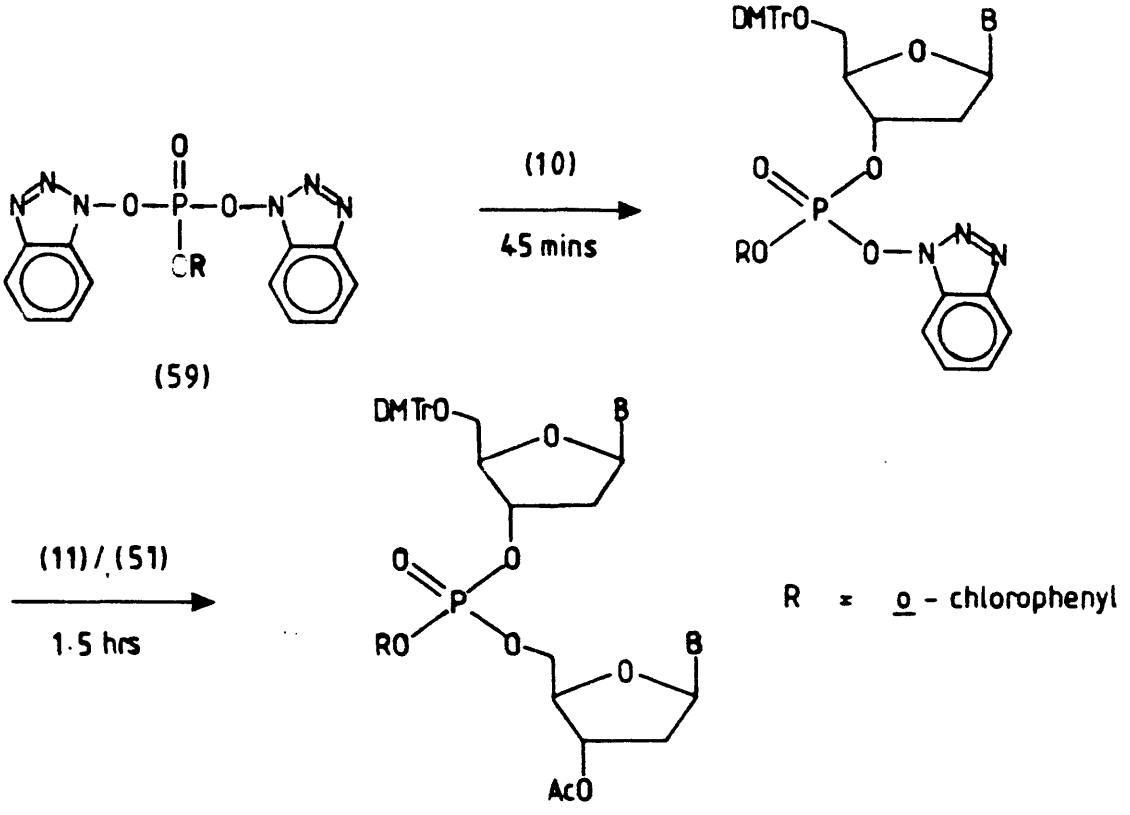
An alternative to the di-triazolide phosphorylating agent (50) has been introduced by van Boom[58] where the O,O,bis-(1-benzotriazoyl) derivative (59) is the phosphorylating agent. This reagent is readily prepared



(59)

R = o - chlorophenyl

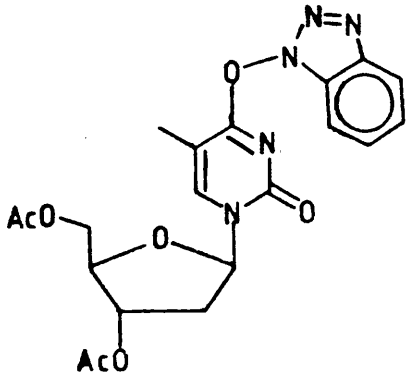
from o-chlorophenyl phosphorodichloridate. Use of (59) in the first phosphorylation step is reported to proceed with high selectivity for monosubstitution, so much so that the second phosphorylation step can be carried out directly, although the presence of 1-methylimidazole (51) is still required, Figure 1.21.



(46)

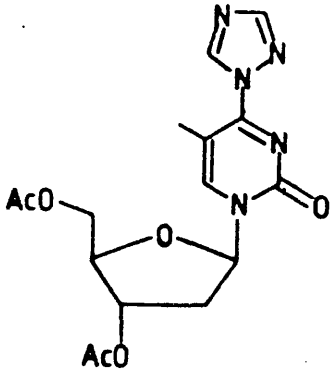
Figure 1.21

Van Boom and his co-workers have stressed that,[59,60] despite its high reactivity in both phosphorylation steps, reagent (59) does not attack and therefore modify base residues. However, Reese and Richards[61] have recently shown that (59) does modify base residues, in particular, guanosine derivatives, to give a complicated mixture of products and thymidine where the modified thymidine derivative (60) was isolated in 85% yield when 3',5'-di-o-acetylthymidine was treated with (59) for 1 hour.



(60)

In comparison, treatment of 3',5'-di-O-acetylthymidine with the di-triazolide phosphorylating agent (50), under the same conditions, takes 6 hours to yield only a 27% conversion into the analogously modified derivative (61).



(61)

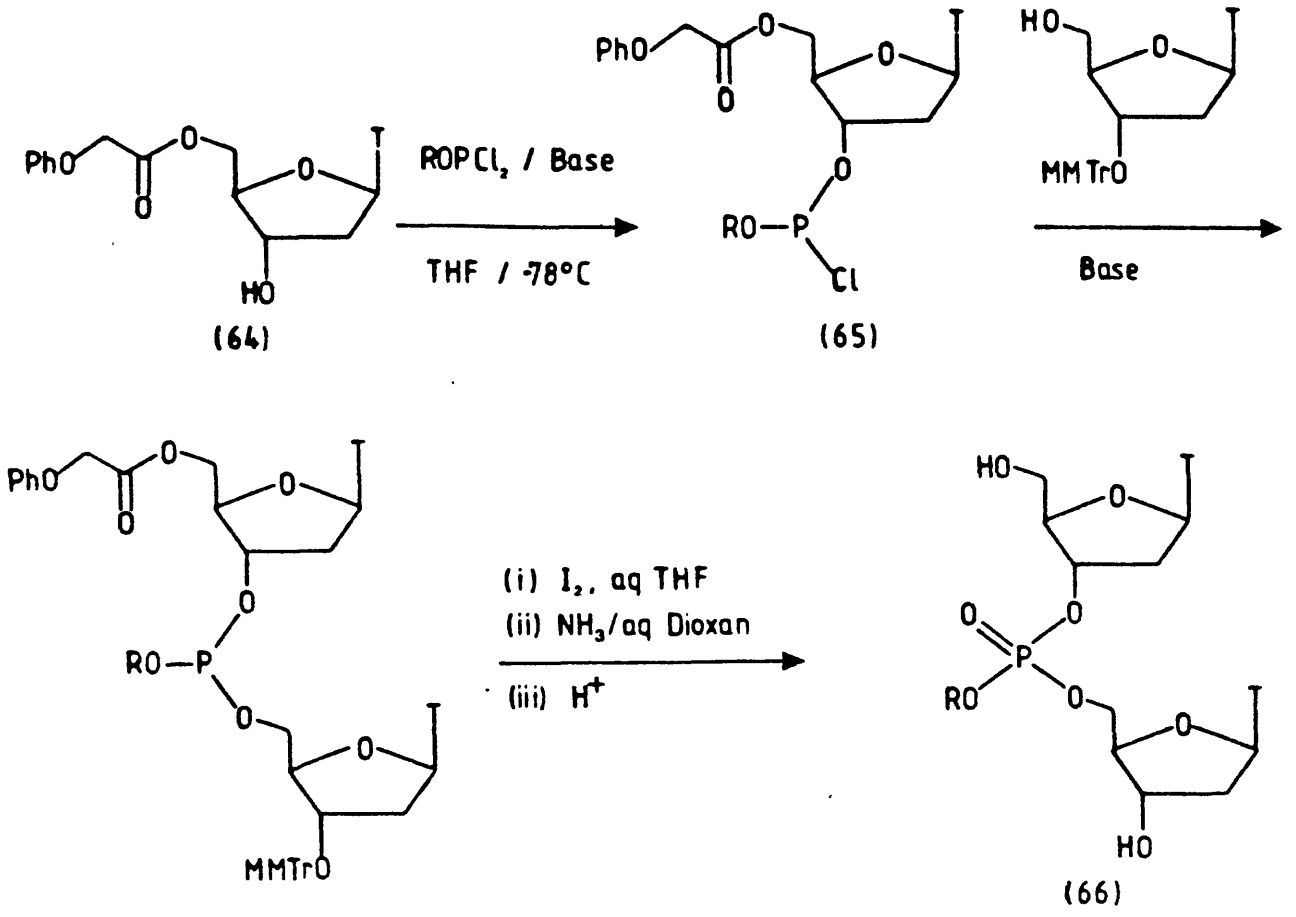
It would therefore appear that for efficient synthesis by the phosphotriester approach with the minimum of side reactions the approach which

employs o-chlorophenyl phosphorodi-(1,2,4-triazolide) (50) as the initial phosphorylating agent is the preferred method of choice.

The Phosphite Triester Approach

The rate of reaction of phosphorylating agents of the type $ROP(O)Cl_2$ and $(RO)_2P(O)Cl$ with the 3'-hydroxyl functions of nucleosides is relatively slow. Advances from this have culminated in the use of the di-triazolide phosphorylating agent (50) which phosphorylates the 3'-hydroxyl function in ~ 60 minutes, but introduces the added complication of base modification which has led to advances in new protecting groups. The belief that this procedure is time consuming and laborious has prompted various research groups to examine the synthesis of oligonucleotides using phosphite triester rather than phosphate triester intermediates, thereby utilising the more rapid reaction rates of phosphorus (III) compounds.

Letsinger and Lunsford[62] synthesised fully protected TpT using o-chlorophenyl phosphorodichloridite (62) as the phosphitylating agent, Figure 1.22. Thus, 5'-O-phenoxyacetylthymidine (64), present in slight excess, when treated with (62) and one molar equivalent of 2,6-lutidine in THF at -78 °C gave the phosphorochloridite (65). After 6 minutes, the 3'-O-monomethoxytritylthymidine was added to give the fully protected phosphite triester. Oxidation with iodine in aqueous THF at -10 °C, followed by removal of the hydroxyl protecting groups gave TpT (66) in 65% yield. The internucleotide protecting groups used were o-chlorophenyl and 2,2,2-trichloroethyl. The phosphorodichloridites (62) and (63) are extremely reactive, so much so that an excess of the 3'-hydroxyl component was used. Inevitably, formation of the (3'-3') symmetrical product was observed. Nevertheless, these workers were able to carry out the stepwise synthesis of $(Tp)_4T$ in this way using 2,2,2-trichloroethyl phosphorodich-



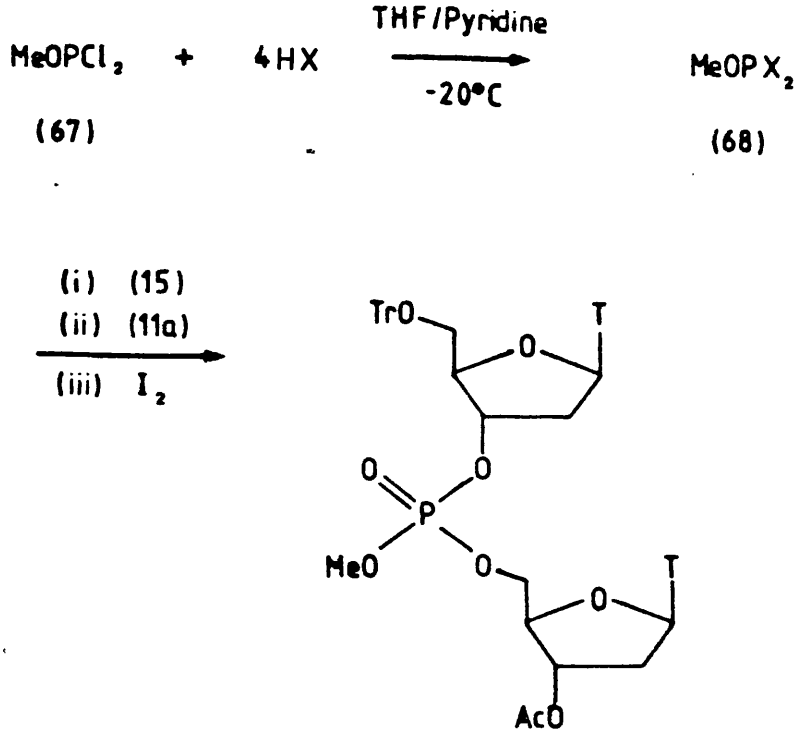
(62) , R = o-chlorophenyl

(63) , R = 2,2,2-trichloroethyl

Figure 1.22

loridite (63) as the phosphitylating agent.

Due to the high reactivity of the phosphorochloridites and their subsequent poor specificity for primary compared to secondary hydroxyls, Fourrey and Shire[63] looked at the possibility of using heterocyclic bases to moderate the reactivity of the phosphitylating species in much the same way as they had in the phosphotriester approach. Four equivalents of the chosen base, Figure 1.23, were added to one molar equivalent of methyl phosphorodichloridite (67) at -20 °C to give, presumably, bis (1,2,4-triazolide)methoxyphosphine (68a) or bis (tetrazolide)methoxyphosphine (68b) After 10 minutes, the mixture was cooled to -78 °C and sequentially



(69)

X = $-N\langle$ (68a) 70%

X = $-N\langle$ (68b) 75%

X = Cl (67) 38%

Figure 1.23

treated with 5'-O- tritylthymidine (15), 3'-O-acetylthymidine (11a) and then warmed to -20 °C before oxidation with iodine. However, the bis-(azolide)methoxyphosphines are unstable towards air oxidation and hydrolysis and, ideally, a stable monofunctional phosphitylating agent was required. In the phosphite triester approach the methyl group is generally used as the internucleotide protecting group which can be selectively removed under mild conditions with thiophenol[30] in Et_3N/Dioxan solution, the mechanism being SN2 attack of thiophenoxide at the methyl carbon. However, due to the poor selectivity for monosubstitution of (67) newly proposed phosphitylating agents with protecting groups removed by

β-elimination (β-cyanethyl)[64] or by reductive elimination (1,1-dimethyl-β,β,β-trichloroethyl)[65] introduced for their greater selectivity for monosubstitution may replace the methyl group in future.

An alternative method of overcoming the problem was introduced by Caruthers and Beaucage[66] by synthesising a new class of nucleoside phosphites. The key intermediates prepared were N,N-dimethylaminophosphoramidites (71a-d). They were prepared as shown in Figure 1.24.

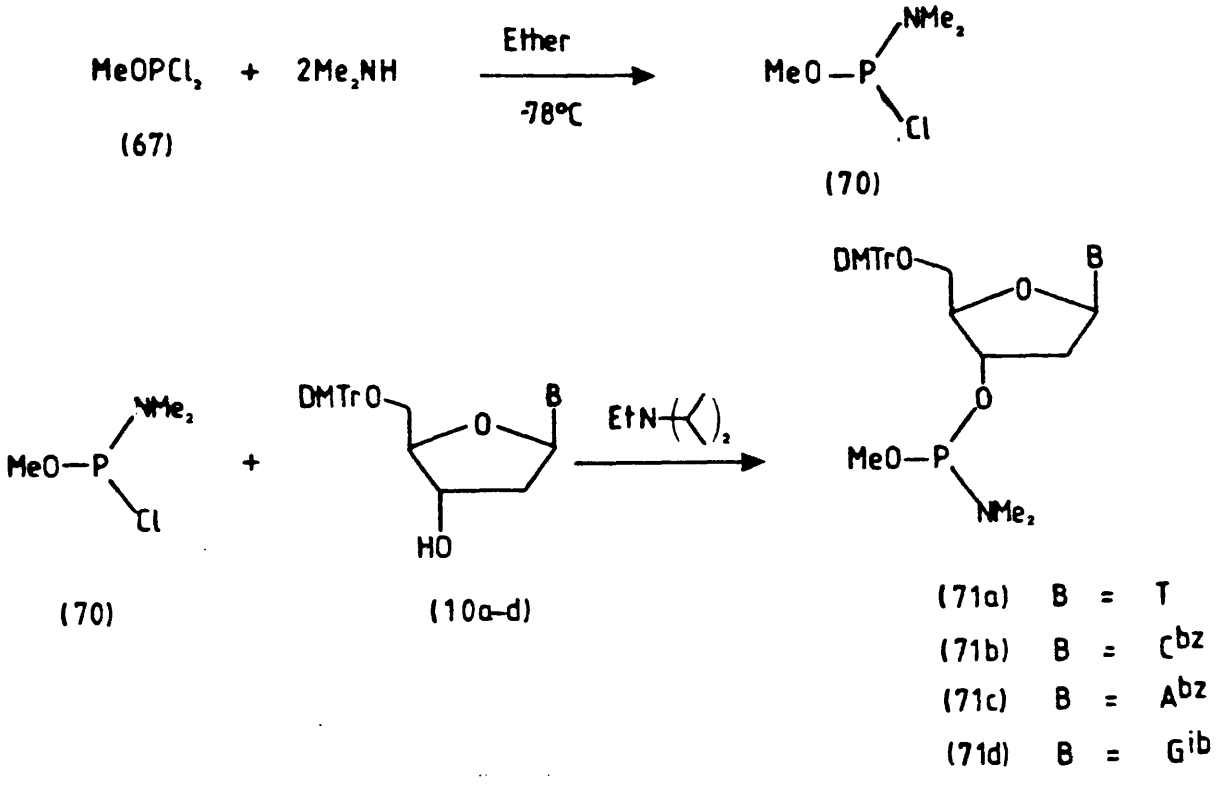


Figure 1.24

Chloro-N,N-dimethylaminomethoxyphosphine (70) is prepared by slow addition of two molar equivalents of dimethylamine at low temperature to methyl phosphorodichloridite (67). Following filtration and distillation (70) is obtained in 71% yield. (70) is a monofunctional phosphitylating agent and can be condensed with the 5'-protected monomer (10) to give (71) which can be isolated by precipitation into cold hexane. The phosphoramidites (71a-d) require activation prior to phosphite formation and this is achieved by addition of tetrazole (72) as the acid catalyst, Figure 1.25.

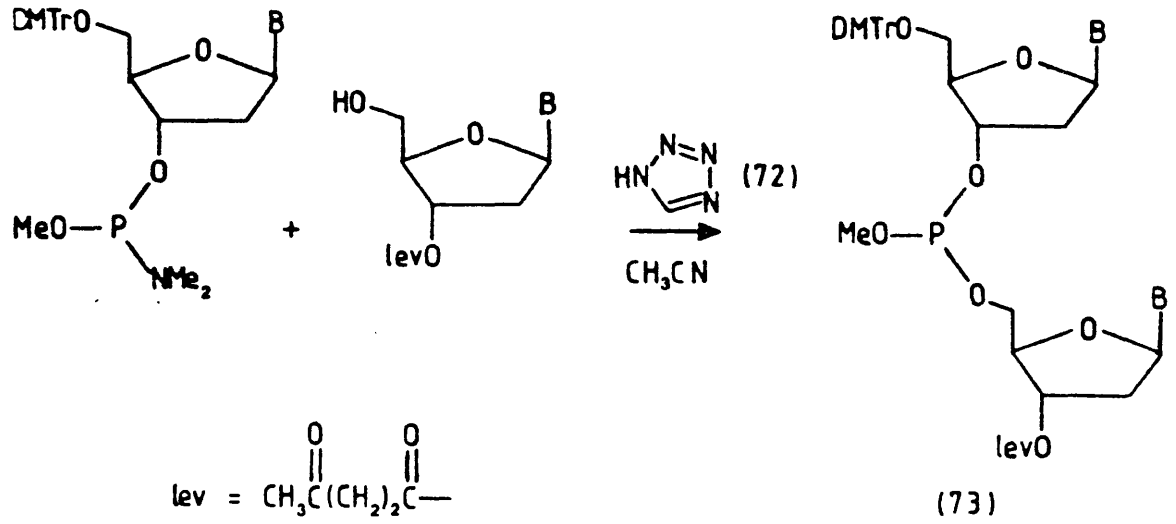
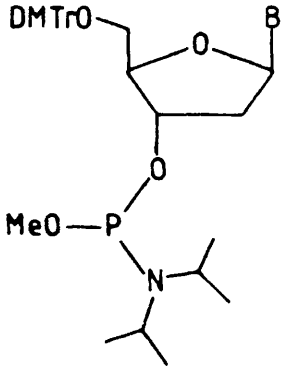
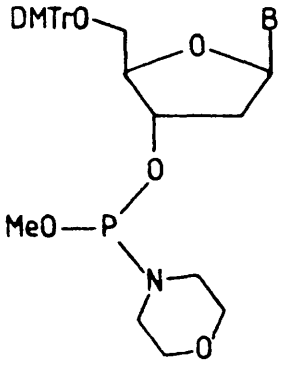


Figure 1.25

The phosphite (73) is rapidly formed under these conditions and can be oxidised to the phosphate triester with iodine in aqueous THF. In a later publication, Caruthers and Mcbride[67] survey the limitations of the phosphoramidites (71) with regard to their sensitivity to hydrolysis. To circumvent this, these workers synthesised two other phosphoramidites (74a-d) and (75a-d) from the preceding chlorophosphines and showed them





(74a) B = T
(74b) B = C^{bz}
(74c) B = A^{bz}
(74d) B = G^{ib}

(75a) B = T
(75b) B = C^{bz}
(75c) B = A^{bz}
(75d) B = G^{ib}

to be much more stable than the dimethyl analogue, to such an extent that they could be purified by column chromatography. However, the preceding chlorophosphines are difficult to prepare and easily react with trace

amounts of water. This problem has been overcome by Caruthers[68] by preparing the phosphoramidites (74) and (75) from the preceding bis-alkylaminophosphines exemplified in Figure 1.26 for compound (74).

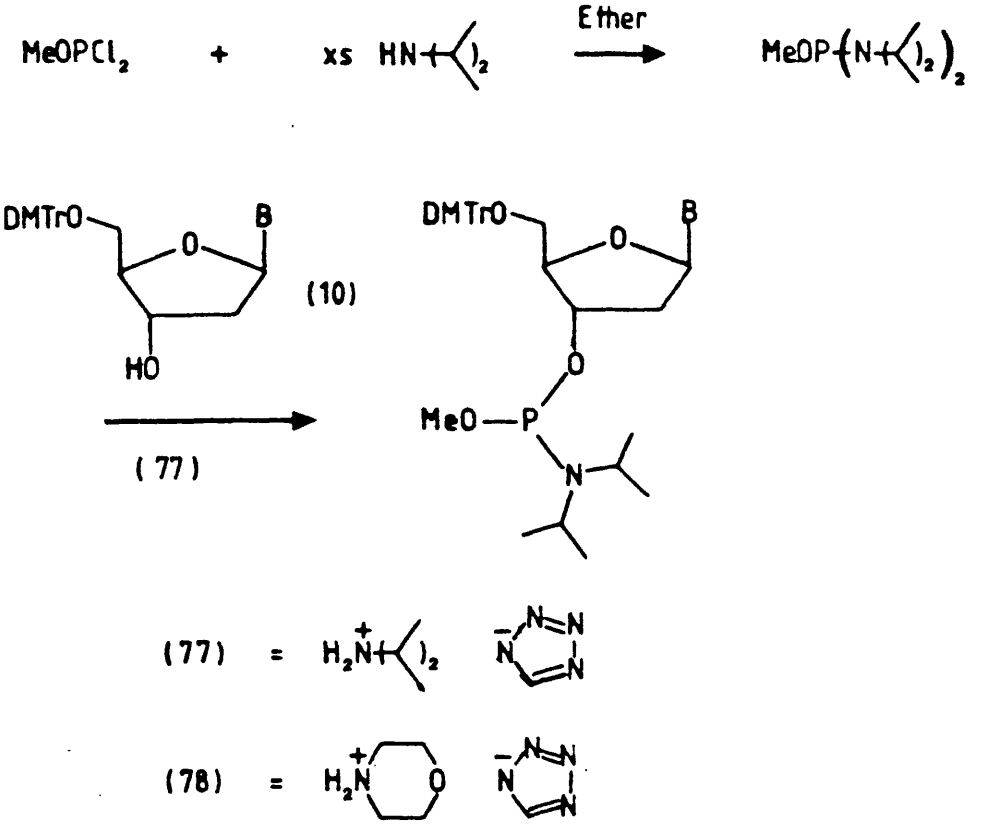


Figure 1.26

The amine salt (77) [and (78) for the morpholino analogue (75)] catalyses the phosphitylation reaction and excellent selectivity for monosubstitution is obtained, the required phosphoramidites (74a-d) and (75a-d) being obtained in 63 - 70% yields, after chromatography.

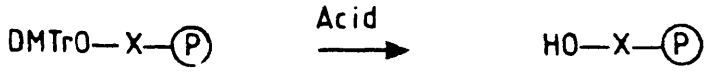
The Solid Phase Method

Both the phosphotriester method and the phosphite triester method, in solution require coupling of the appropriately protected monomer unit, containing a 3'-phosphate, or a 3'-phosphoramidite, with another with a free 5'-hydroxyl group. Following coupling [and oxidation if P(III) route] the products are ultimately separated by chromatography. Long oligomers therefore, require several deprotections, phosphorylations (or phosphitylations) and chromatographic purifications. This is time consuming and

labour intensive.

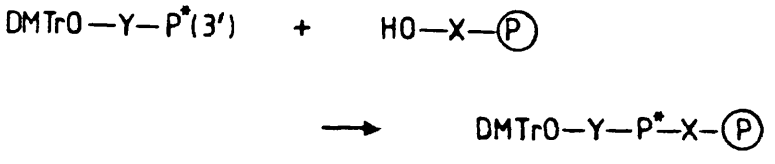
The solid phase method[69,70] brings advantages of speed, microscale operation, labour reduction and automation. The principle of solid phase is that a 5'-protected deoxyribonucleoside is attached, through its 3'-hydroxyl position, to an insoluble, macromolecular, inorganic polymer. Chain assembly is effected by alternating terminal 5'-deprotection reactions and coupling reactions, either phosphotriester or phosphoramidite Figure 1.27. In both cases excess reagent is added to force reactions to

1. Terminal Deprotection



2. Wash

3. Couple



4. Wash (or oxidation followed by wash if phosphoramidite route)

5. Capping.

x,y = Protected Nucleosides

(P) = Polymer Support

P* = Phosphate or Phosphite derivative

Essential steps of one cycle in chain assembly

Figure 1.27

completion and unreacted components are removed by washing of the inorganic support with an appropriate solvent. A capping step is involved so as to render any 5'-hydroxyl groups that failed to couple unreactive towards further phosphorylation (or phosphitylation) such that only the required sequence is formed; this is generally carried out by acylation. Cycles

of synthesis are continued until the oligomer of required length and sequence is obtained at which point the oligomer is cleaved from the support, protecting groups removed and the deprotected product purified.

The Support

Currently, five types of polymer supports are available, Figure 1.28.

	Phosphotriester	Phosphite Triester
Polystyrene	√	√
Silica gel	√	√
Glass beads (CPG)	√	√
Polyamide/Kieselguhr	√	
Cellulose paper	√	

√ = Suitability of each method to each support.

CPG = Controlled Pore Glass

Figure 1.28

Silica gel and CPG are useful on a microscale in both manual and mechanised operation because of low loading (30-70μ mol/g), rigidity and resistance to swelling. Polystyrene and Kieselguhr/polyamide (a composite of polydimethylacrylamide gel embedded in macroporous Kieselguhr) are better on a larger scale as their ability toswell allows better accessibility of sites when highly loaded with material (> 100μ mol/g). Cellulose paper has the advantage of simultaneous synthesis of a large number of oligonucleotides on a microscale.

Universally a succinate link is used to join the first residue to the support via a long chain alkylamine "spacer", Figure 1.29. The terminal deprotection, step 1, Figure 1.27, is achieved by a weak protic acid (trichloroacetic or dichloroacetic). The deprotection is extremely

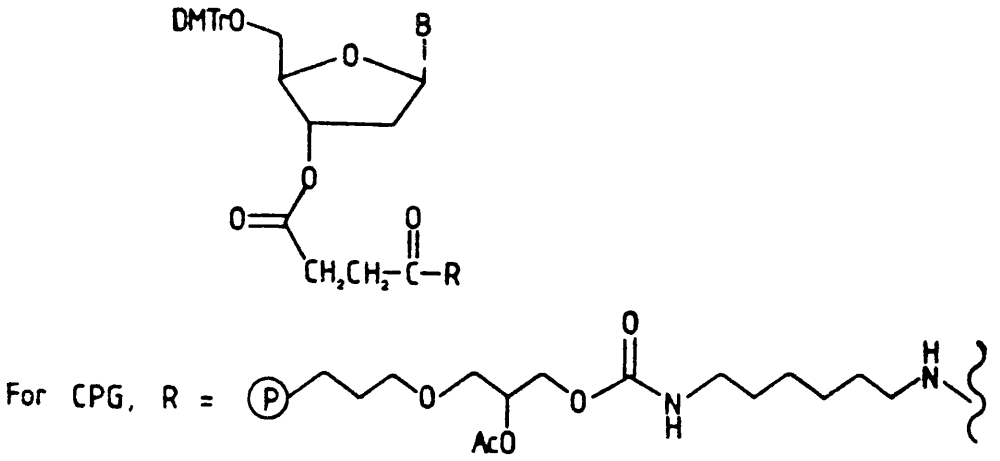


Figure 1.29

rapid and the dimethoxytrityl cation released is highly chromophoric and can be used as an indirect assay procedure.

At the end of the synthesis, it is essential that the protecting groups are removed in the correct order. The phosphate protecting groups are removed first (oximate or thiophenolate) to form the phosphodiesters. Ammonia is used to cleave the N-acyl groups and finally, acid is used to remove the terminal DMTr group. The link to the support is cleaved at the same time as the o-chlorophenyl groups (by oximate) in the phosphotriester route, but during ammonia treatment in the phosphoramidite route.

Purification and Sequence Analysis

Oligonucleotides are, essentially, polyanions containing lipophilic nucleobases and therefore anion-exchange and reversed-phase chromatography are useful for isolation and analysis of a particular product.

Anion-exchange chromatography depends upon the adsorption and desorption of the anionic solute on a cationic stationary phase. Therefore desorption of a mixture of polyanionic solutes will depend upon the net charge of each polymer and in the case of oligonucleotides, the chain length. In reversed phase chromatography the stationary phase is non polar and the mobile phase is polar and hydrophobic interactions determine the velocity of migration along the stationary phase. High performance, or high pressure liquid chromatography (HPLC) has become a standard

purification procedure in chemical DNA synthesis. The chromatographic interactions are no different to those found in other chromatographic systems, the difference being that the column inlet pressures are often very high. Ultra-violet detectors are employed as the oligonucleotides have high extinction coefficients in the range 250 - 280 nm.

Following isolation, from the HPLC column, to obtain sequence information the wandering spot analysis is probably most effective. It is based on the characteristic mobility shifts of sequential partial degradation products of an oligonucleotide on a two dimensional chromatogram obtained by high voltage electrophoresis (hve) and homochromatography. The oligonucleotide is labelled at the 5'-end using T4 polynucleotide kinase and $\{\gamma\text{-}^{32}P\}$ ATP and then hydrolysed using snake venom phosphodiesterase (SVPD). During the hydrolysis reaction aliquots are withdrawn and saved from which a mixture is prepared containing all partial degradation products in equal amounts. This is subjected to hve on a cellulose acetate strip at pH 3.5. At this pH the difference in protonation of the four heterocyclic bases is maximal. Therefore, the mobility of a component of the mixture in an electric field depends on base content. The consecutive degradation products after hve are sorted in a second chromatographic step according to their chain length. The material from the hve cellulose acetate strip is transfered to a DEAE-cellulose thin layer plate. The plate is developed with a partial hydrolysate of RNA in the mobile phase. The basis of the separation is the homochromatography of the radioactive degradation products with non-labelled RNA oligomers of the same length. The RNA mixture proceeds via displacement of short oligomers by longer ones having more negative charges that bind more strongly to the DEAE-cellulose. Therefore, short oligomers migrate faster than longer ones.

CHAPTER 2

ARBUSOV REACTIONS ON MODEL NUCLEOSIDE PHOSPHITES

The Arbusov Reaction and The Iodine Oxidation

The reaction of trialkyl phosphites with alkyl halides or elemental halogen to give dialkyl alkylphosphonates and phosphoryl halides respectively, is known as the Michaelis-Arbusov reaction[72], Figure 2.1.

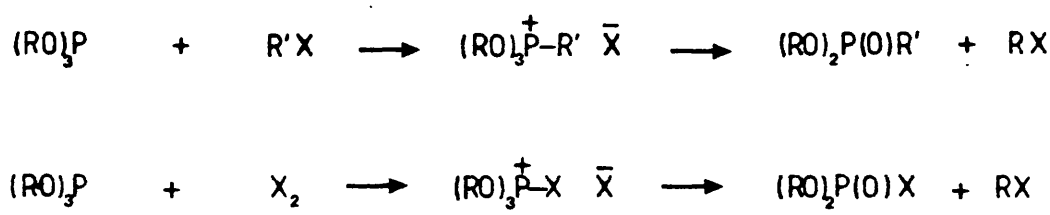
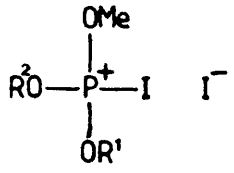


Figure 2.1

The reaction involves conversion of a coordination- three phosphorus compound, bearing a P-OR group, into a coordination - four compound with a P:O group. Phosphinites ($ROPR_2$) and phosphonites ($(RO)_2PR$) undergo an identical reaction to form phosphine oxides and phosphinates respectively. The first stage of the reaction is nucleophilic attack of the trivalent phosphorus compound on the substrate (R'X or X_2) to form a phosphonium salt, displacing X^-, which, in the second stage of the reaction removes one of the alkyl substituents from the phosphorus, releasing the alkyl halide RX, and generating the extremely strong phosphorus - oxygen double bond; the driving force for the reaction. Phosphonates are important as they are reagents in the Wittig-Horner[73] and Wadsworth-Emmons[74] olefin forming reactions and phosphoryl halides have found value in peptide bond formation.[75]

In the phosphite triester route for synthesis of oligonucleotides, once the phosphite has been formed from a phosphorochloridite or phosphoramidite precursor, the phosphate triester is obtained by oxidation with $I_2/THF/H_2O$. This reaction presumably, initially proceeds via the formation of the phosphonium salt (79) as does the first stage of the Arbusov reaction.



R^1, R^2 = 5'- and 3'-protected nucleoside residues

(79)

In the case of simple trialkyl phosphites, the second stage of the reaction involves rapid attack at carbon by iodide to give the dialkyl phosphoroiodidate[76a 76b]. However, in the presence of water,(79) must undergo hydrolysis faster than dealkylation,since no dealkylation products are observed. The mechanism of the aqueous iodine oxidation is obscure as, from simple arguments overall inversion of configuration at phosphorus would be expected, in that the formation of (79) from nucleophilic attack of the phosphite on iodine would be expected to occur with retention of configuration. Subsequent hydrolysis of (79) via an envisaged in-line SN2 type mechanism would proceed predominantly with inversion of configuration, resulting in overall inversion. However, Cullis[77] has shown that the aqueous iodine oxidation of dinucleoside phosphites proceeds with retention of configuration at phosphorus thereby confirming that the mechanism is more complex than this simple argument implies. The phosphitylating agents, be they phosphorochloridites or phosphoramidites, in the phosphite triester route are extremely sensitive to hydrolysis. It is desirable, therefore, particularly during solid phase synthesis, to avoid the introduction of water at any stage, especially with regard to minimizing the number of steps in an automated procedure. The use of an aqueous solvent therefore demands a drying step afterwards, prior to subsequent phosphitylation, normally effected by repeated washing with an anhydrous organic solvent. Although non-aqueous oxidation procedures have been

reported,[78 79] the aqueous iodine procedure still remains the preferred method of choice.

The t-Butyl Substituent and the Need for a t-Butyl-Containing Phosphitylating Agent

In a series of Arbusov reactions on several mixed trialkyl phosphites Trippett[76b] observed the second stage of the Arbusov reaction to show SN1 character, Figure 2.2. Where the phosphite contained a t-butyl group as

Phosphite	Products with	
	MeI	Br_2
$(EtO)_2P-OBu^t$	$(EtO)_2P(O)Me$	$(EtO)_2P(O)Br$

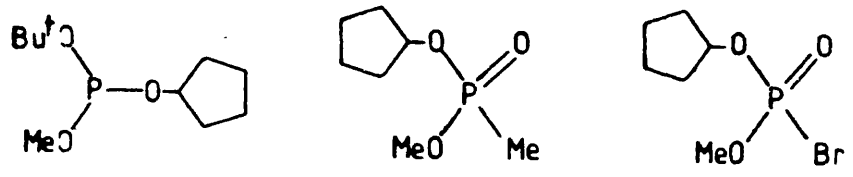
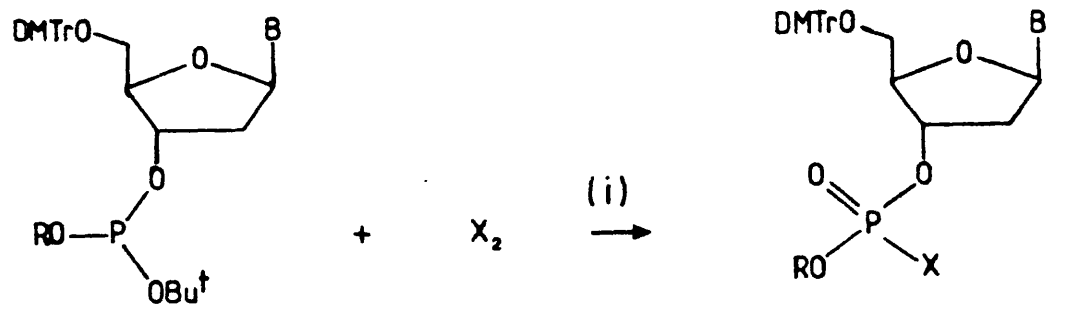


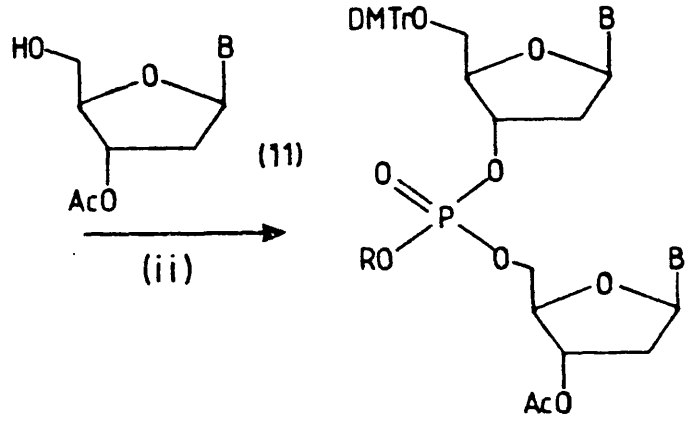
Figure 2.2

one of the substituents, then t-butyl halide was always eliminated in preference to methyl, ethyl or cyclopentyl halide. This behaviour has also been reported by Mark[80] in a series of reactions of mixed phosphites with hexachlorocyclopentadiene as the alkyl halide. This observation that loss of t-butyl halide is favoured reinforces the view that the second stage of the reaction shows SN1 character although formation of a discrete carbonium ion is unlikely.

This experimental observation can be used therefore, to propose a very general, but novel scheme, for the synthesis of oligonucleotides, Figure 2.3.



(80) (81)



(82)

Figure 2.3

A trialkyl phosphite, bearing a 5'-O- protected nucleoside residue and the t-butyl substituent, such as (80), when treated with elemental halogen (Cl_2, Br_2, I_2) may be expected to undergo an Arbusov reaction as described, to form a phosphoryl halide (81), with loss of t-butyl halide. Subsequent condensation with the 3'-O-protected monomer (11) under suitable, hitherto unknown conditions, should result in the formation of the fully protected phosphate triester (82).

A number of potential advantages are apparent from a route such as this.

(i) The Arbusov reaction, in addition to providing a phosphorylating agent (81) (X = Cl, Br) also simultaneously effects oxidation of the phosphite (80) under non-aqueous conditions.

(ii) Arbusov reactions on trialkyl phosphites with elemental halogen proceed very readily, even at low temperature. Therefore, step (i) in Figure 2.3, would be expected to proceed efficiently with rapid formation of (81).

(iii) Phosphorochloridates of the type (81, X = Cl), have previously been used in the synthesis of oligonucleotides by the phosphotriester approach. Michelson and Todd[29] in the first phosphotriester synthesis used phosphorochloridates. Eckstein and Rizk,[32] when examining the 2,2,2-trichloroethyl phosphate protecting group used phosphorochloridates as initially did Reese[25] when investigating the phenyl protecting group. However, these were discarded as phosphorodichloridates were their precursors which led to disubstitution problems resulting in formation of the undesired (3'-3') symmetrical product (Figure 1.15, page20). In Figure 2.3, however, the phosphorochloridate (81, X = Cl) or phosphorobromidate (81, X = Br) formed, would by virtue of its mode of formation, be monofunctional, therefore eliminating the possibility of (3'-3') symmetrical phosphate formation.

(iv) As no (3'-3') symmetrical phosphate can be formed, no isolation of the phosphorylating agent (81) for the second coupling is required, as is necessary in the phosphotriester route when o-chlorophenyl phosphorodi-(1,2,4-triazolide) (50) is used as the initial phosphorylating agent (Figure 1.17 page 22).

However, in addition to the potential advantages brought by this method there are several criteria that need to be met before it can be accepted as a viable alternative to the already well established phosphotriester and phosphoramidite methodologies.

(i) In addition to the 5'-O-protected nucleoside residue and the t-butyl substituent present in the phosphite (80) (Figure 2.3), a further grouping R is required, a grouping that will not compete in the Arbusov reaction and will remain intact throughout, thereby serving as the internucleotide protecting group.

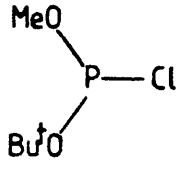
(ii) Prior to the formation of (80), an isolable, stable, monofunctional phosphitylating agent is required, already containing the protecting group R and the t-butyl group.

(iii) Phosphorochloridates (81, X = Cl) and phosphorobromidates (81, X = Br) do not react rapidly with alcohols in the presence of just base. For a competitive route, therefore, step (ii) in Figure 2.3, needs to be rapid.

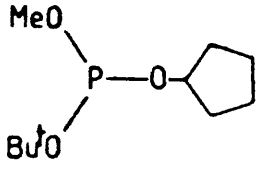
(iv) It must be ensured that under the conditions required to carry out the Arbusov reaction, the various hydroxyl and amino protecting groups remain intact.

(v) The method needs to be applicable to solid phase synthesis.

It was initially intended, therefore, to investigate the viability of this route with both phosphate protecting groups used at present - the methyl group and the o-chlorophenyl group. The phosphorochloridite incorporating the methyl protecting group would therefore be t-butyl methyl phosphorochloridite (83).

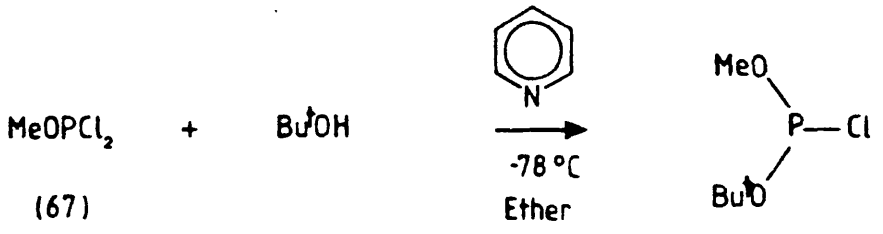


(83)



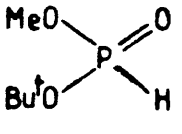
(84)

Although this compound has been reported as an "in-situ" intermediate in the preparation of t-butyl cyclopentyl methyl phosphite (84), it was not isolated. The preparation and isolation of this compound were attempted by slow addition of an ethereal solution of t-butanol to a solution of methyl phosphorodichloridite and pyridine in ether at low



(83)

temperature. After the addition period of 90 minutes the mixture was warmed to room temperature. The ^{31}P n.m.r. spectrum showed a signal at +164.9p.p.m. due to the required phosphorochloridite (83). However, due to the high reactivity of (67), and although the alcohol is tertiary and therefore hindered, the mixture was contaminated with approximately 16% (estimated by ^{31}P n.m.r.) of a species with a chemical shift of + 130.3 p.p.m., assigned to di-t-butyl methyl phosphite, resulting from disubstitution. Consequently, the mixture also contained approximately 15% of the starting material (67, ^{31}P n.m.r. chemical shift + 183.7 p.p.m.). Attempted isolation of (83) by filtration, celite aided, and removal of excess solvent gave a cloudy oil. The ^{31}P n.m.r. spectrum of the crude material contained a major component having a chemical shift of + 3.0 p.p.m., with no absorption present at +164.9 p.p.m. due to the required phosphorochloridite (83). The compound obtained is assigned as t-butyl methyl phosphite on the basis of literature values for this compound[81] and



t-Butyl Methyl Phosphite

from the P-H coupling (J_{P-H} = 690Hz) observed from the partially decoupled ^{31}P n.m.r. spectrum and from the ^{1}H n.m.r. spectrum. This compound, resulting from hydrolysis of the required phosphorochloridite was also obtained when similar experiments were conducted using acetonitrile and THF as solvent. The phosphorochloridite (83) therefore, was found not to be isolable but was sufficiently pure and stable in solution for phosphite formation when simple mixed trialkyl phosphites were being prepared. This is satisfactory as the mixture of phosphites obtained can be separated by distillation. This is not the case with nucleoside phosphites of the type (80), R = Me. Therefore, the phosphitylating agent needs to be free from impurities so that clean conversion into the phosphite can be obtained prior to the Arbusov reaction.

An alternative approach was to use a variation of the phosphoramidite method. This entailed the preparation of t-butyl methyl N,N-dimethyl-phosphoramidite (85), as shown in Figure 2.4.

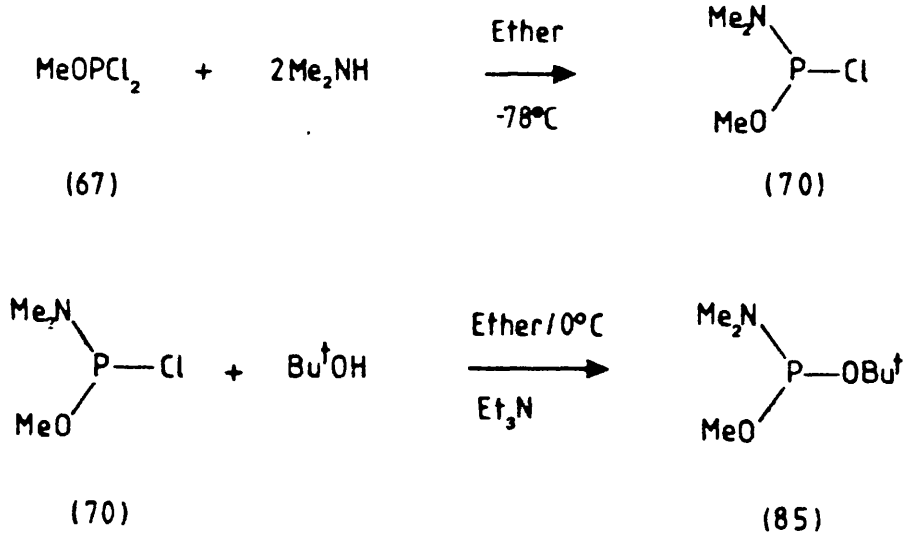


Figure 2.4

Initially, chloro-N,N-dimethylaminomethoxyphosphine (70) was prepared by the method of Caruthers[66] and isolated in 68% yield. The phosphoramidite (85) was obtained by treatment of (70) with t-butanol

and four molar equivalents of triethylamine in 40% yield. It was envisaged, therefore, to carry out the phosphite formation as in the phosphoramidite route, Figure 2.5.

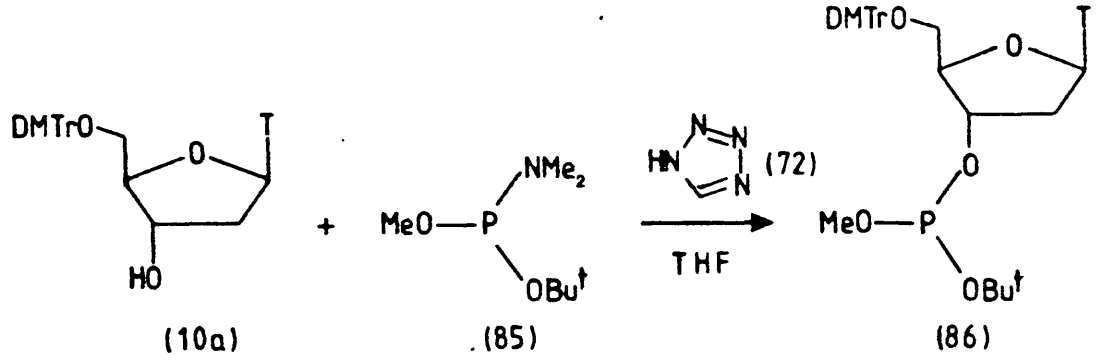
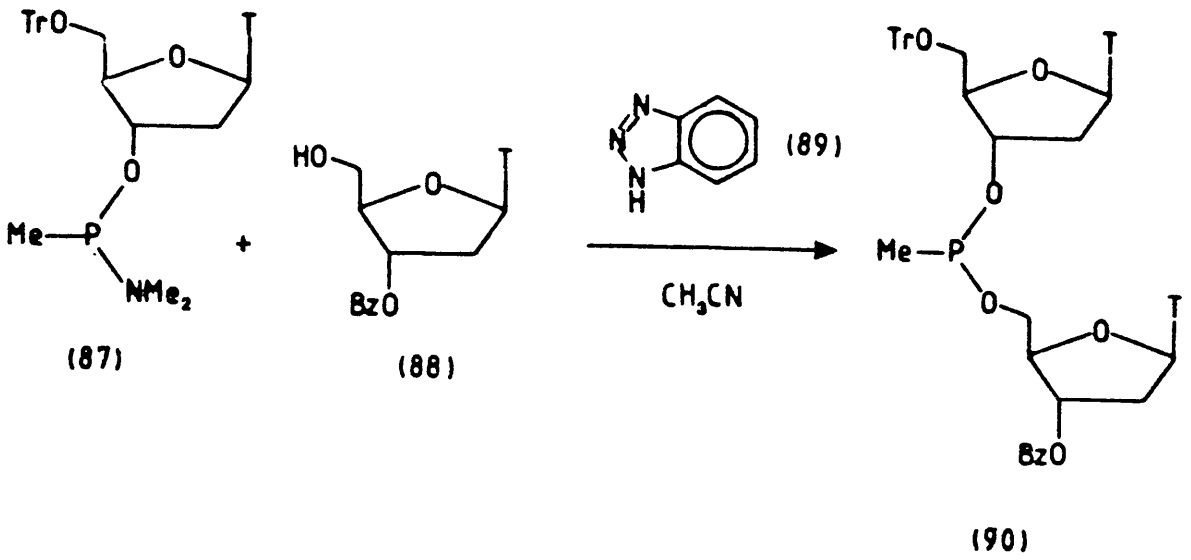


Figure 2.5

Tetrazole (72; 1.1 molar equivalents), resublimed prior to use, was added to a solution of the phosphoramidite (85) and 5'-O-dimethoxytrityl-thymidine in THF solution at room temperature. After 15 minutes, the ^{31}P n.m.r. spectrum showed two major phosphorus containing components having chemical shifts of + 134.9 p.p.m., presumably due to the required phosphite (86), and at + 3.4 p.p.m. due to the hydrolysis product . The diastereoisomers of the phosphite (86) were not resolved, resulting in the signal at 134.9 p.p.m. being broad. However, it is not advised to carry out phosphoramidite reactions of this sort in solution since they are subject to an acid catalysed ligand exchange reaction resulting in a statistical mixture of all possible phosphites. This behaviour has been observed by Engels[82] in the preparation of dinucleoside phosphonites from the phosphonamidite (87); Figure 2.6. To form the phosphonite (90) the phosphonamidite (87) is activated with 1H - benzotriazole (89) and treated with the 3'-O-protected monomer (88). Short reaction times are advised as, after 15 minutes, 6% of (92) is formed from the slow acid catalysed ligand exchange reaction. This reaction is reported as being much more rapid with tetrazole (72) as activating agent. Hoffman[83]


TrO
T
O
O
Me—P
NMe₂
(87)
+
HO
T
O
BzO
(88)
N
N
N
H
(89)
CH₃CN
TrO
T
O
O
Me—P
O
O
T
BzO
(90)

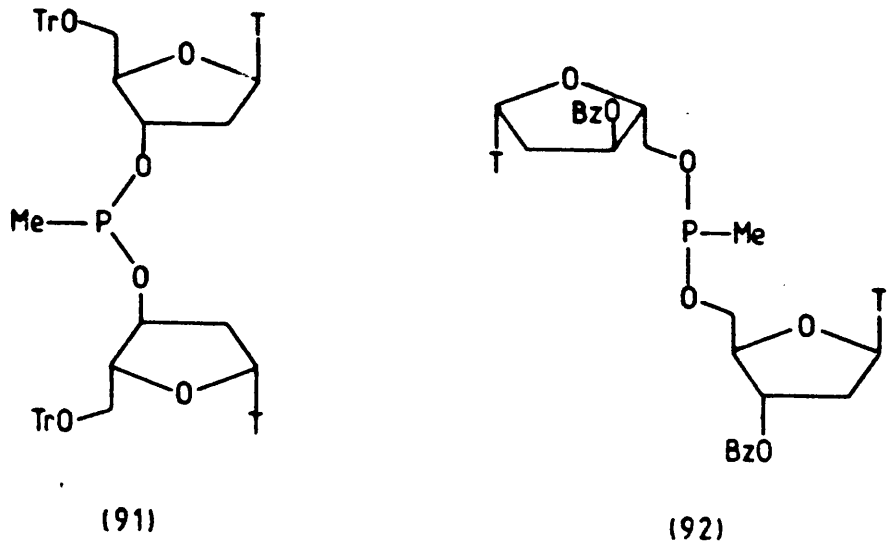

TrO
T
O
O
Me—P
O
O
TrO
T
(91)
O
BzO
T
O
P—Me
O
O
T
BzO
(92)

Figure 2.6

has observed similar disproportionation reactions for dialkyl alkylphosphinites and for the reorganisiation of trialkyl phosphites Moedritzer established a strong catalytic effect.[84] This is a process not observed in solid phase synthesis, a fact that suggests the mechanism may be bimolecular. Since the alcohol to be phosphitylated and the phosphite subsequently obtained are attached to an inorganic support this "bimolecular" process is therefore unable to operate.

A further,potentially more serious problem is apparent with regard to the t-butyl substituent. After 70 minutes a further absorption was present in the ^{31}P n.m.r. spectrum at + 7.0 p.p.m. as a minor component of the mixture. This species was not isolated but is thought to be due to an acid catalysed elimination of isobutylene from the t-butyl substituent, shown in Figure 2.7.

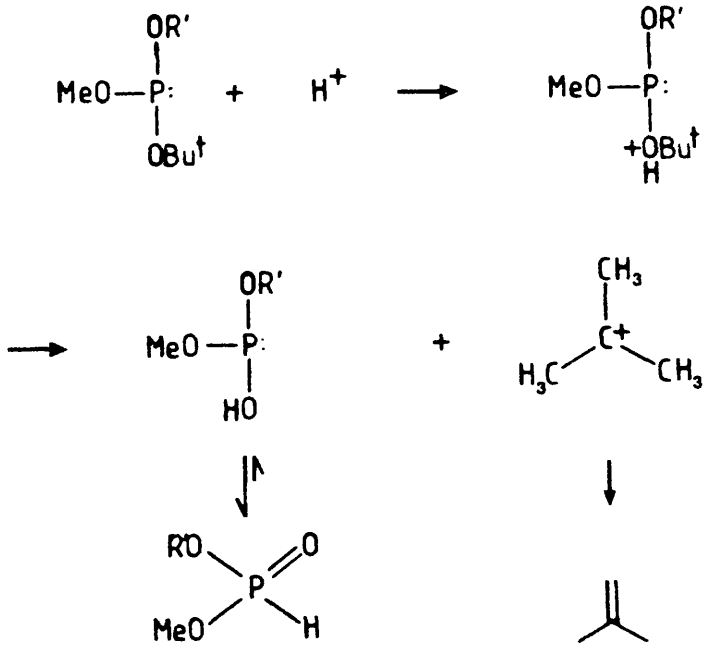
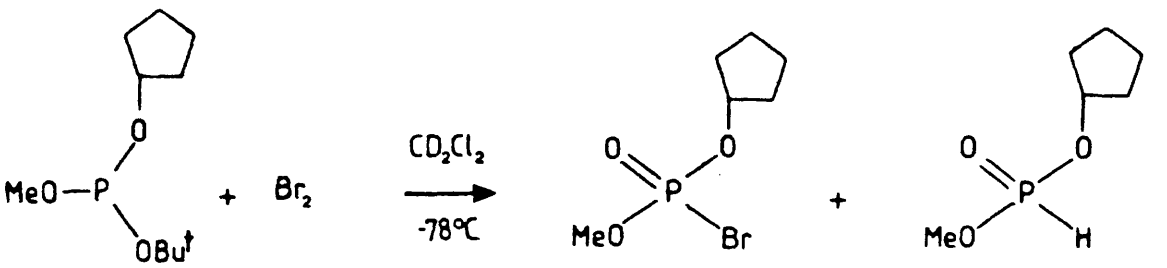


Figure 2.7

Goldwhite[85] has observed similar behaviour in the acid catalysed elimination of isobutylene from tri-t-butyl phosphite and Mark[86] showed that isobutylene can be eliminated from tri-t-butyl phosphite upon warming to 50 ^{o}C without the presence of an acid catalyst. This was not viewed as being too serious a problem in the phosphite forming reaction but, as model experiments showed, it became a problem during the subsequent Arbusov reaction; Figure 2.8. Thus,when t-butyl



(84) (93) (94)

Figure 2.8

cyclopentyl methyl phosphite (84) was treated with one molar equivalent of bromine in deuterated methylene chloride at low temperature the ^{31}P n.m.r. spectrum showed two absorptions at -5.6 p.p.m. (55%) and + 10.6 p.p.m. (45%, based on ^{31}P n.m.r.). The component at -5.6 p.p.m. is cyclopentyl methyl phosphorobromidate (93) and the component at + 10.6 p.p.m. was shown to contain a P-H bond from the doublet in the partially decoupled ^{31}P n.m.r. spectrum and from the proton n.m.r. spectrum (J_{P-H} = 680 Hz). This is therefore postulated to be cyclopentyl methyl phosphite (94), the acid catalyst thought of as being due to HBr present in the bromine. Similar experiments carried out in ether, THF and acetonitrile as solvent produced similar results.

From the experiments conducted it was concluded that the t-butyl substituent was too sensitive to use as required in spite of its unique potential in the Arbusov rearrangement. Therefore, for the required criteria to be met an alternative group was sought that would realise

the same properties as the t-butyl group in the Arbusov rearrangement without being as sensitive to side reactions. Therefore, several model experiments were carried out on simple mixed phosphites in an attempt to satisfy the required condition.

Alternative "Leaving Groups" in the Arbusov Rearrangement

(a) Dimethyl 2-methoxyethyl phosphite (95)

In view of the apparent SN1 character of the second stage of the Arbusov reaction it was anticipated that the methoxyl oxygen in the phosphite (95), which is β to the carbon atom to bear the positive charge, Figure 2.9, would provide neighbouring group participation to stabilise the

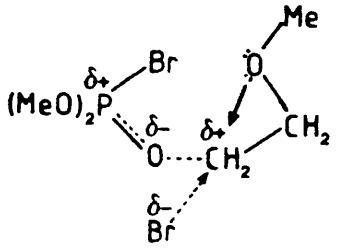
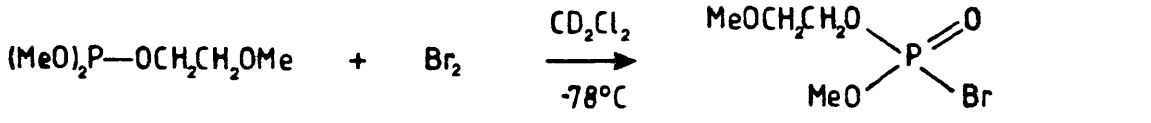


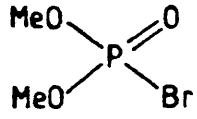
Figure 2.9

developing positive charge. As a result, therefore, it would encourage selective removal of the 2-methoxyethyl group.



(95) (96)

δ^{31}P n.m.r (CD_2Cl_2) −5·9 p.p.m



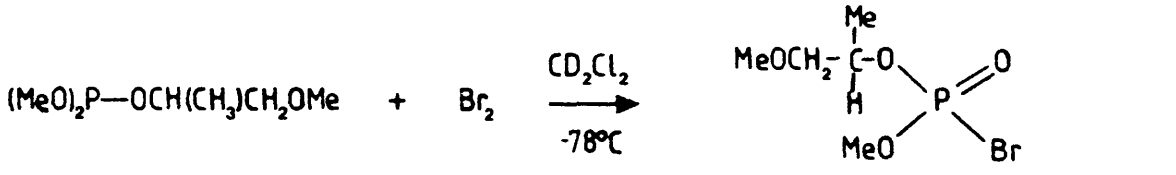
(97)

δ^{31}P n.m.r (CD_2Cl_2) −4·03 p.p.m

When dimethyl 2-methoxyethyl phosphite (95) was treated with bromine at low temperature and the ^{31}P n.m.r. spectrum recorded there was a major absorption at -5.9 p.p.m. with a minor component at -7.2 p.p.m. There was no signal present due to dimethyl phosphorobromidate (97), the chemical shift of which $\left[\delta\ ^{31}P(CD_2Cl_2)\right.$ - 4.03 p.p.m.$\left.\right]$ had been confirmed by treatment of trimethyl phosphite with bromine under the same conditions, the product that would arise from exclusive loss of the 2-methoxyethyl group. The ^1H n.m.r. spectrum contained a singlet at δ 2.6 due to the methyl protons of methyl bromide with no observable signal at δ 3.34 due to the methylene protons α to the bromine in 2-methoxyethyl bromide although this region of the spectrum was quite complex. This data is consistent with virtual exclusive loss of methyl bromide in the Arbusov reaction to give 2-methoxyethyl methyl phosphorobromidate (96).

(b) Dimethyl 1-methoxypropan-2-yl phosphite (98)

In addition to the argument used for the phosphite (95) an extra methyl



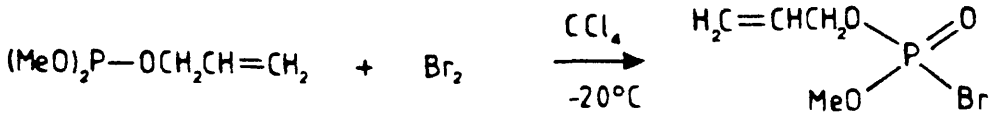
(98) (99)

δ^{31}P n.m.r (CD_2Cl_2) −6·65 p.p.m

group was incorporated into this phosphite on the carbon atom that is to bear the partial postive charge in the second stage of the Arbusov reaction, to provide a positive inductive effect in addition to the envisaged NGP effect displayed in Figure 2.9. Dimethyl 1-methoxypropan-2-yl phosphite (98) when treated with bromine at low temperature produced a ^{31}P n.m.r. spectrum containing one peak only with a chemical shift of -6.65 p.p.m. No absorption was present at - 4.03 p.p.m. characteristic

of dimethyl phosphorobromidate (97) and the ^1H n.m.r. spectrum of the reaction mixture contained the signal at δ 2.6 due to methyl bromide as before and no signal at δ 4.1. characteristic of the proton α to the bromine in 2-bromo-1-methoxypropane, which is the alkyl halide that would result from loss of the 1-methoxypropan-2-yl grouping. Therefore this reaction had proceeded with exclusive loss of methyl bromide to give 1-methoxypropan-2-yl methyl phosphorobromidate (99). It was expected that loss of the new group would be observed at least to some extent during this reaction since the centre of interest is secondary. This is not consistent with the observations of Trippett[76b] in phosphites where methyl is competing against a secondary substituent in similar reactions. It is plausible, therefore, to suggest that the β methoxy substituent is exerting a negative inductive effect and not an NGP effect, therefore destabilising the developing positive charge to such an extent that SN2 attack at the methyl group, resulting in loss of methyl bromide, is preferred.

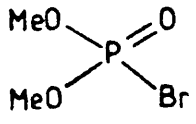
(c) Allyl dimethyl phosphite (100)



(100) (101)

δ^{31}P n.m.r(CCl_4) −6.1 p.p.m

+



(97)

δ^{31}P n.m.r(CCl_4) −4.2 p.p.m

This reaction was carried out in carbon tetrachloride as solvent. Phosphites are known to react with carbon tetrachloride to give trichloromethylphosphonates.[87] This reaction requires more severe conditions than the cold,short,reaction times used here; hence, it was not anticipated to compete. The allyl group was introduced so as to delocalise the partial positive charge over the π system, Figure 2.10, under conditions where SN1 character is shown, thereby enticing the allyl system

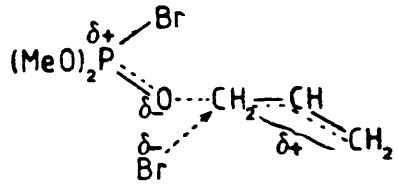


Figure 2.10

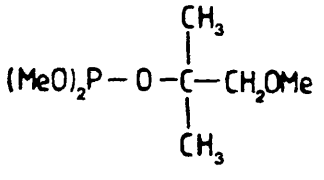
to depart as allyl bromide. Following the addition of bromine to allyl dimethyl phosphite (100) at -20 °C, the ^{31}P n.m.r. spectrum contained two signals at -4.2 p.p.m. due to dimethyl phosphorobromidate (97) and at -6.1 p.p.m. in the ratio of 2:1 (by ^{31}P n.m.r.). Calculations from the ^{1}H n.m.r. integral values (MeBr, δ 2.6 vs $BrCH_2CH = CH_2$ δ 3.9, methylene protons α to bromine) and statistical alterations indicated the reaction had proceeded with a 4:1 preference for loss of the allyl group, allyl methyl phosphorobromidate (101) being the minor phosphorus component. This observation is not confirmation of an SN1 mechanism involving the allyl group as although SN1 rates are increased over alkyl substrates due to resonance stabilisation of the carbonium ion, and even more so when substituents are incorporated into the 1 and 3 positions that can stabilise a positive charge; the rate of SN2 displacements at allylic positions is also increased due to resonance interaction with the double bond in the transition state. SN2' displacements can also occur at the γ carbon of allylic substrates but generally only when steric crowding is introduced at the α position. Therefore, although loss of

the allyl halide was favoured under these conditions it was not exclusive, methyl bromide being observed in the ^1H n.m.r. spectrum and allyl methyl phosphorobromidate being observed in the ^{31}P n.m.r. spectrum. From this result therefore, indications were that the allyl group could not be satisfactorily used as required.

(d) Cyclopentyl 1-methoxy-2-methylpropan-2-yl methyl phosphite (103)

The 1-methoxy-2-methylpropan-2-yl substituent is a tertiary group containing an electron-with drawing substituent. This was introduced so as to discourage the acid catalysed elimination of the substituent as in the t-butyl case by reducing the possibility of a positive charge developing at the tertiary centre. This would mean, with regard to the Arbusov reaction, the mechanism of departure of the new group would not be as high in SN1 character as was the t-butyl group, although it was hoped to be sufficient so as to compete successfully against methyl.

The phosphite initially required therefore was dimethyl 1-methoxy-2-methylpropan-2-yl phosphite (102). The preparation of this compound



(102)

was attempted in the same manner as the preceding phosphites using the method of Imaev[88] by heating trimethyl phosphite with one molar equivalent of the required alcohol in the presence of the sodium alkoxide, Figure 2.11. In all previous cases methanol was removed as it was formed after which the crude reaction mixture was distilled under reduced pressure to give the required phosphite. The analogous reaction using 1-methoxy-2-

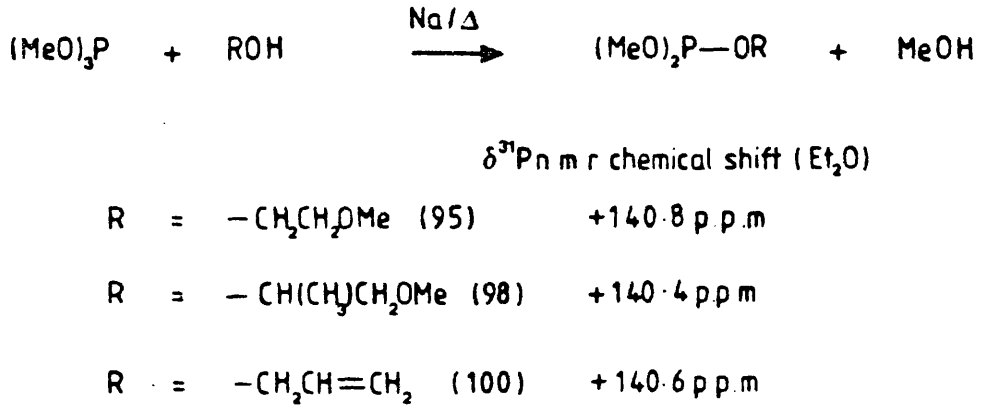
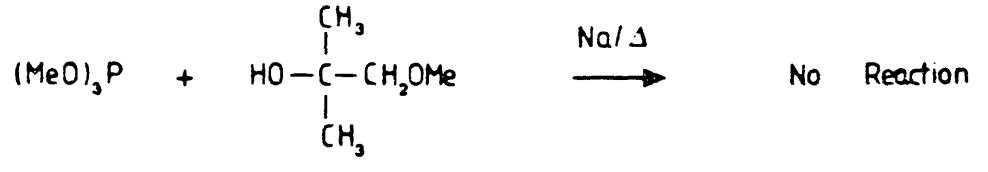
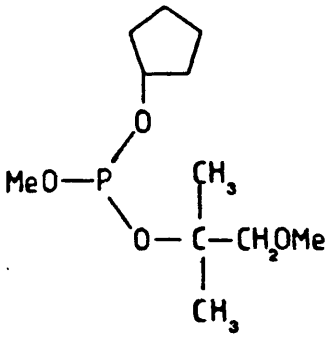


Figure 2.11

methylpropan-2-ol as the alcohol was attempted. The reaction mixture refluxed at 108 °C, (b.pt trimethyl phosphite = 111 °C, b.pt 1-methoxy-2-

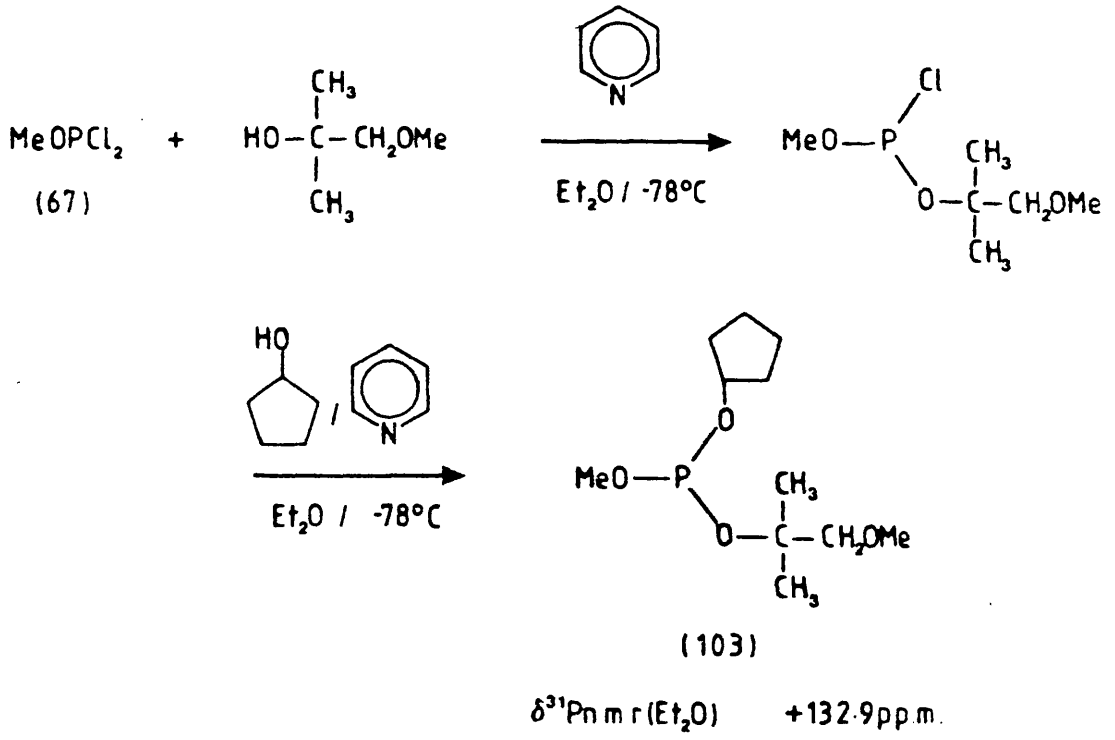


methylpropan-2-ol = 114 °C) and no methanol (b.pt 64 °C) was generated as in the previous three cases. The reaction mixture was refluxed for a further several hours and no detectable reaction occurred, the only signal present in the ^{31}P n.m.r. spectrum after this time was due to trimethyl phosphite [δ ^{31}P (ether) + 140.8 p.p.m.]. The reaction, therefore, readily proceeds with primary and secondary alcohols but not at all with tertiary alcohols. Indeed, in the original publication, Imaev does not report the use of tertiary alcohols, only primary and secondary. However, the two other groups present in the phosphite need not both be methyl groups. They had been used in the initial studies for simplicity and ease of preparation. Ideally, therefore, the model phosphite required was cyclopentyl 1-methoxy-2-methylpropan-2-yl methyl phosphite (103).



(103)

This was prepared by the method of Trippett[76b], Figure 2.12, by sequential treatment of methyl phosphorodichloridite (67) with 1-methoxy-2-methylpropan-2-ol and pyridine and then with cyclopentanol and pyridine in ether at low temperature. The pyridinium hydrochloride was removed



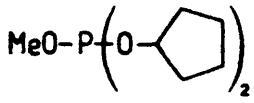
(103)

δ^{31}P n.m.r. (Et_2O) +132.9 p.p.m.

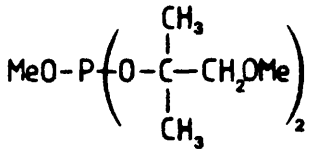
Figure 2.12

by filtration through a short column of activated alumina. The solvent was removed under reduced pressure to leave a cloudy oil. The ^{31}P n.m.r. spectrum showed a major absorption at + 132.9 p.p.m. (ether) due to (103)

and two minor absorptions at + 137.9 p.p.m. (14% by ^{31}P n.m.r.) and at + 129.3 p.p.m. (16% by ^{31}P n.m.r.). These two components are assigned to di-cyclopentyl methyl phosphite (104) and di-1-methoxy-2-methylpropan-2-yl methyl phosphite (105) respectively resulting from disubstitution due

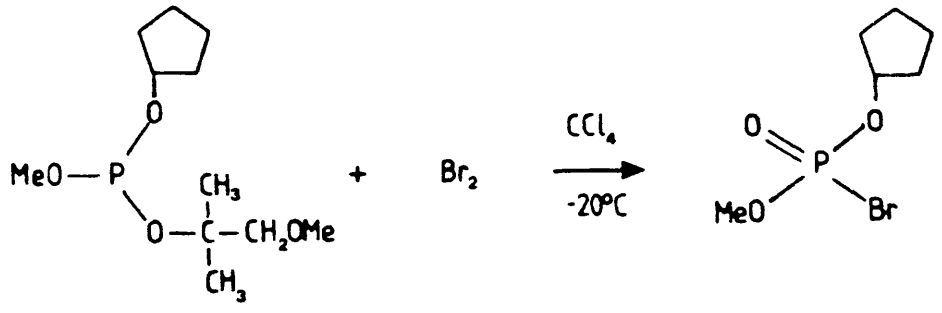


(104)



(105)

to the high reactivity of (67). Distillation under reduced pressure succeeded in removing (104) although a small amount of (105, 14% by ^{31}P n.m.r.) was still present. This phosphite would, of course, also undergo the Arbusov reaction but it was anticipated that the product from it would not confuse the required experimental observation since the required phosphite (103) was present as 86% (by ^{31}P n.m.r.) of the mixture.



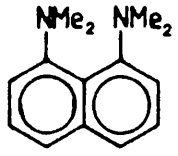
(103)

(93)

$\delta^{31}P$ n.m.r (CCl_4) -7.8 p.p.m

One molar equivalent of bromine was added to the phosphite (103) in carbon tetrachloride at -20 °C. The ^{31}P n.m.r. spectrum showed two major absorptions at -7.8 p.p.m. and +6.7 p.p.m. in the ratio of 41% and 59% (by ^{31}P n.m.r.) respectively. The species at -7.8 p.p.m. was confirmed as cyclopentyl methyl phosphorobromidate (93) by reaction of

t-butyl cyclopentyl methyl phosphite (84) with bromine under the same conditions, a species of identical chemical shift being obtained. The species at +6.7 p.p.m. was shown to contain a P-H bond from the presence of a doublet in the partially decoupled ^{31}P n.m.r. spectrum and also by the presence of an absorption at δ 11.8 in the ^{1}H n.m.r. spectrum, ascribed to the low field half of the P-H doublet (J_{P-H} = 680 Hz). It was anticipated that the species present at +6.7 p.p.m. was cyclopentyl methyl phosphite (94) obtained by acid catalysed elimination of the tertiary substituent. The evidence obtained in this experiment, therefore, implied that the 1-methoxy-2-methylpropan-2-yl substituent was displaying the sensitive properties observed for the t-butyl group. A subsequent experiment was conducted under almost identical conditions, the modification being that the reaction was carried out in the presence of four molar equivalents of 1,8-bisdimethylaminonaphthalene or "proton sponge".



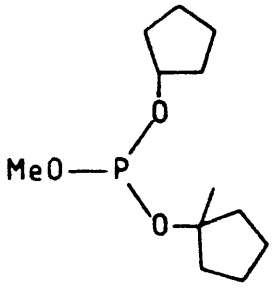
"Proton Sponge"

This compound has been reported by Alder[89] as being a very strong base, more so than normal aliphatic amines, due to relief of steric strain upon protonation and it is only weakly nucleophilic due to steric reasons. It was envisaged, therefore, that the proton source reputed to be responsible for the decomposition of the phosphite would be removed in the presence of "proton sponge". The ^{31}P n.m.r. spectrum of the analogous reaction in the presence of "proton sponge" showed a dramatic decrease in the intensity of the signal at +6.7 p.p.m. in the ^{31}P n.m.r. spectrum, although its presence was not eliminated completely. This underlines, therefore,

the very sensitive nature of the tertiary substituents employed.

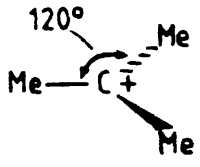
(e) Cyclopentyl 1-methylcyclopentyl methyl phosphite (106)

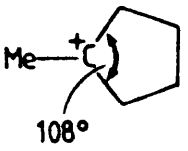
The argument used for the introduction of the 1-methoxy-2-methyl-propan-2-yl group, with regard to Figure 2.7, is also pertinent to the 1-methylcyclopentyl group, utilised as an alternative tertiary substituent. Therefore, cyclopentyl 1-methylcyclopentyl methyl phosphite (106) was prepared in an identical manner to (103), Figure 2.12, and obtained in



(106)

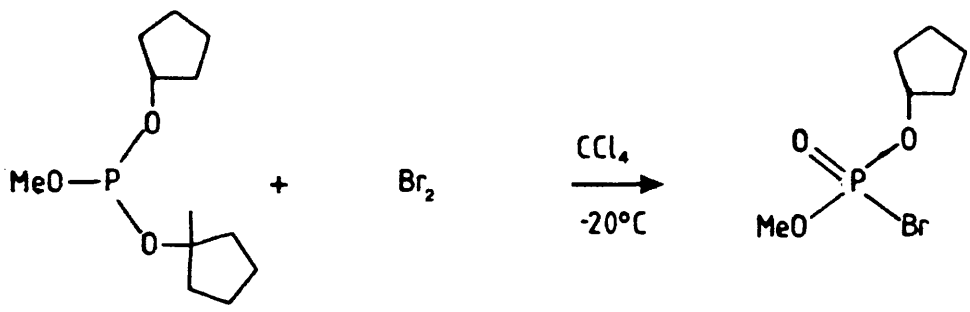
45% yield. The partially formed carbonium ion generated in the acid catalysed elimination of the tertiary substituent would be less stable than in the case of the t-butyl group, since a considerable amount of distortion is required to accommodate the preferred arrangement of 120^{o} bond angles due to the confinements of the five membered ring, it





preferring a bond angle of 108^{o}. 1-Methylcyclopentanol was prepared by treatment of cyclopentanone with methylmagnesium iodide.[90] However, the maximum reported yield is only 22% and indeed, 1-methylcyclopentanol

was obtained in only 17% yield. Purification was effected by vacuum distillation and then by sublimation, the major impurities prior to this being unreacted cyclopentanone, evident by the carbonyl stretch in the infra red at 1740 cm^{-1} and olefinic species, characterised by the olefinic stretch at 1660 cm^{-1} in the infra red and a multiplet at δ 5.2 in the ^1H n.m.r., these presumably being due to dehydration of the alcohol at some point during work-up. Following the addition of one molar equivalent of bromine to the phosphite (106) the ^{31}P n.m.r. spectrum showed



(106) (93)

$\delta^{31}P$ n.m.r(CCl_4) -7.8 p.p.m

a major phosphorus absorption at +7.0 p.p.m. and a peak of lesser intensity at -7.8 p.p.m. due to the required phosphorobromidate (93). The species at +7.0 p.p.m. was again shown to be a P-H containing compound due to the doublet being observed in the partially decoupled ^{31}P n.m.r. spectrum and the ^1H n.m.r. spectrum (J_{P-H} = 680 Hz). Other minor species were also present in the mixture having chemical shifts between -10.8 p.p.m. and -14.3 p.p.m. These absorptions were in the region of the spectrum characteristic of tetraalkyl pyrophosphates formed, presumably, due to the presence of trace amounts of water as shown in Figure 2.13. It must be emphasised that the reason several pyrophosphate species were present arises from the presence in the starting material of phosphites derived from disubstitution of methyl phosphorodichloridite (67) even following distillation. Repetition of the reaction in the presence of "proton sponge" yielded a similar result to that obtained with the phosphite (103).

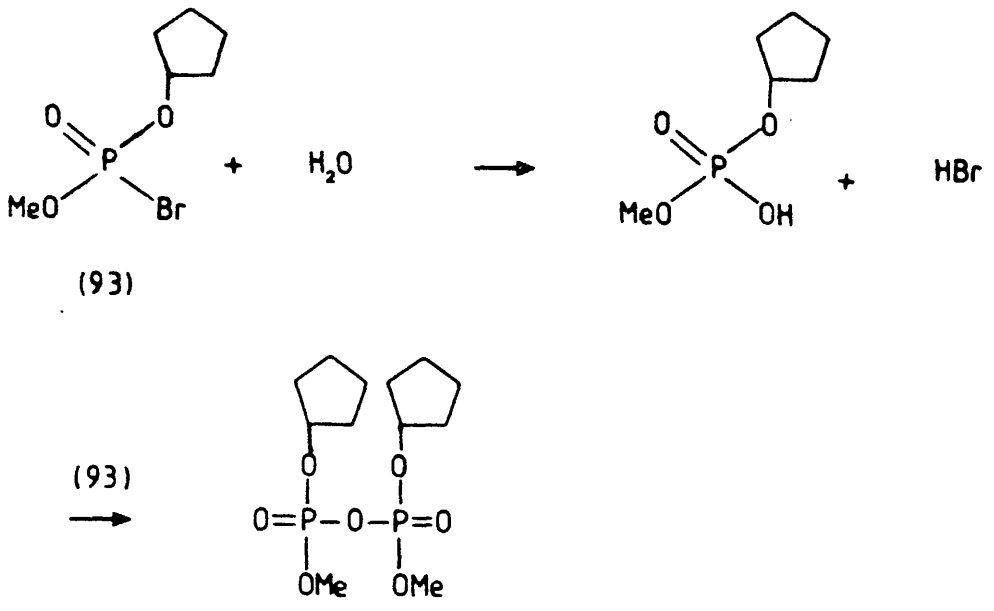


Figure 2.13

A dramatic decrease in the intensity of the signal at +7.0 p.p.m. was observed revealing the phosphorobromidate (93) to be the major phosphorus containing component present in the reaction mixture.

Conclusion

The reactions described represent the attempted exploitation of the SN1 character that the Arbusov reaction, under certain conditions, can show, culminating in the investigation of tertiary substituents.

Subsequent experiments in which concentration, solvent and stoichiometries or reagents were varied did not produce any better results and, although potentially useful results had been achieved with model compounds, to satisfy criterion (ii) (page 43) required a stable, isolable monofunctional phosphitylating agent. The indications were that this condition could not be met with t-butyl as one of the substituents and since the 1-methoxy-2-methylpropan-2-yl and the 1-methylcyclopentyl groups had not demonstrated any advantages with regard to stability over the t-butyl

group, it was concluded that any attempted preparation of a phosphitylating agent containing either of these two groups would prove to be just as unrewarding.

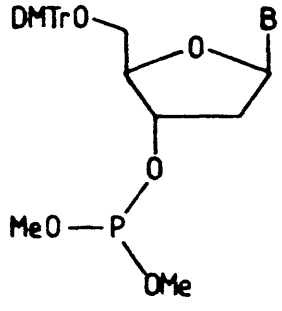
CHAPTER 3

ARBUSOV REACTIONS ON NUCLEOSIDE-CONTAINING PHOSPHITES AND SUBSEQUENT COUPLING REACTIONS IN SOLUTION

Dimethyl Phosphorochloridite as the Phosphitylating Agent

Although the second stage of the Arbusov reaction has been shown to display SN1 character when tertiary substituents are attached, at the opposite extreme it presumably displays largely SN2 character when a methyl group is lost. Therefore, an investigation in which the SN2 character of the second stage of the reaction could be exploited was proposed.

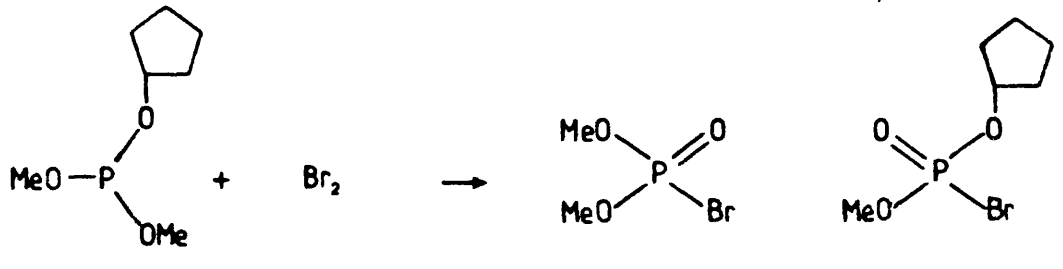
In addition, as only model compounds had been used up to this point, with the cyclopentyl group emulating the nucleoside residue, it was considered prudent to establish how reliable a model the cyclopentyl group was. Therefore, if the methyl group was to remain as the internucleotide protecting group, the phosphite required was 5'-O-dimethoxytrityl-2'-deoxyribonucleoside-3'-O-(dimethyl phosphite) (107).



B = T (107a)
B = C^{bz} (107b)
B = A^{bz} (107c)
B = G^{ib} (107d)

(107)

Although Trippett[76b] had shown that the cyclopentyl analogue of (107), cyclopentyl dimethyl phosphite (108) proceeds in the Arbusov reaction with loss of both cyclopentyl bromide (97) and methyl bromide (93), Figure 3.1,



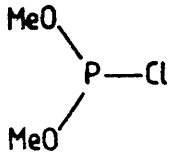
(108) (97) (93)

40% 60%

Figure 3.1

it was considered that this was no guarantee that a similar result would be obtained when cyclopentyl was replaced by a nucleoside residue.

To investigate this point required the preparation of (107), the precursor to which is the dimethyl phosphorochloridite (109).



(109)

This compound has previously been prepared by Lippmann[91] by a disproportionation reaction of phosphorus trichloride and trimethyl phosphite. However, five fractional distillations were required to give only a 6% yield of the required product, still contaminated with 4% of trimethyl phosphite. Ramirez[92] has also prepared (109) by a similar procedure needing only two fractional distillations to obtain pure material although a yield was not quoted. Therefore, (109) was prepared by the slow addition of 2.2 molar equivalents of trimethyl phosphite to phosphorus trichloride, with stirring, at 0 ^{o}C. After the addition time of one hour

$$PCl_3 + 2{\cdot}2\,(MeO)_3P \xrightarrow[\text{r.t. 12hours}]{\text{0°C 1hour}} 3\,(MeO)_2PCl$$

(109)

the mixture was stirred overnight at room temperature. At this point the ^{31}P n.m.r. spectrum showed two major absorptions at +169.9 p.p.m. due to (109), and at +141.1 p.p.m., due to unchanged trimethyl phosphite. A minor absorption was also present at +181.1 p.p.m. due to methyl phosphorodichloridite (67). Two fractional distillations under reduced pressure and one at atmospheric pressure, b.pt (109) = 96^{o}-100^{o} C at 760mm/Hg, succeeded in producing an 11% yield of [109, δ ^{31}P n.m.r. (CH_2Cl_2) +169.4 p.p.m.]. The very low yield is caused by the fact that during distillation several fractions need to be taken, even though a fractionating column was

used, as the boiling points of (67, 92°-94° C) and (109, 96°-100° C) are so similar that separation of the two is difficult. Consequently, in addition to pure fractions of (109), several fractions containing (109) contaminated with small amounts of (67) were collected which could be redistilled.

The proposed reaction sequence is shown in Figure 3.2. Dimethyl phosphorochloridite (109) with one molar equivalent of the 5'-O-protected nucleoside derivative (10 a-d) in the presence of base would be expected to form the phosphite (107a-d). Subsequent reaction with bromine, it was

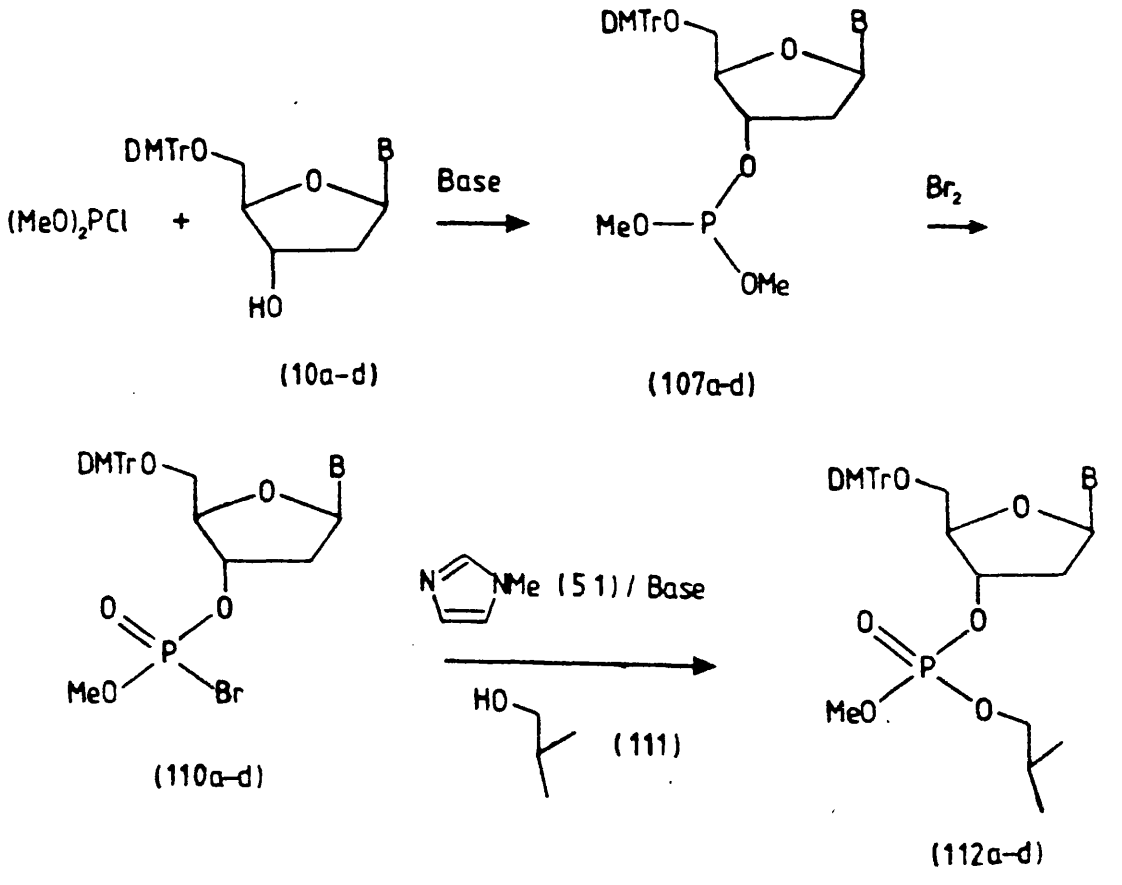


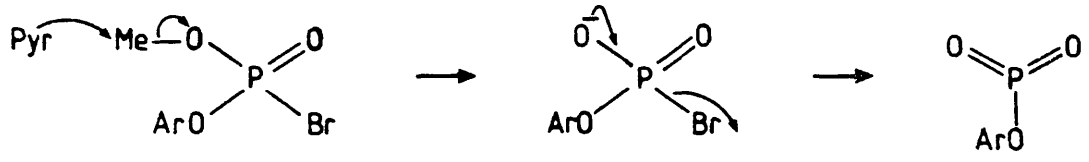
Figure 3.2

hoped would proceed with loss of methyl bromide, as shown, to produce the phosphorobromidate (110a-d). In a one pot procedure, addition of isobutanol (111), used here as a model for the 3'-O-protected nucleoside residue, in the presence of the activating agent 1-methylimidazole*(51) was expected

*The reason for the choice of 1-methylimidazole as activiating agent is discussed in Chapter 4.

to result in the formation of the trialkyl phosphate (112a-d). ^{31}P n.m.r. spectroscopy is ideal for monitoring a reaction sequence of this kind since phosphorus species of the type in Figure 3.2 have characteristic chemical shifts. In addition, as with the model compounds, the presence of dimethyl phosphorobromidate (97), which would result from loss of the nucleoside residue in the Arbusov reaction, would be easily recognisable due to its known chemical shift of -4.0 p.p.m.

In this one pot sequence, several reagents are present at one time. Consequently, there are several sources of potential trouble. Thuong and Chassignol[93] observed that when a phosphorobromidate bearing a methyl substituent is generated in pyridine solution demethylation occurs, Figure 3.3. The demethylated species obtained (113) is reported to phosphorylate



(113) (114)

Ar = o-chlorophenyl

Figure 3.3

a 5'-O-protected nucleoside residue readily to give a phosphate diester. This reaction could go via the metaphosphate (114) which, due to its high reactivity, might undergo unwanted side reactions. The nucleoside residues (10a-d) are hygroscopic and therefore need to be rendered anhydrous prior to use. It is common practice to azeotrope them dry with anhydrous pyridine. As a gum is always obtained when this procedure is carried out, residual pyridine is invariably present throughout the reaction sequence.

It is strongly advised to use no more than a stoichiometric amount of bromine in these reactions as it has been demonstrated[94] that a pyridine/Br_2 charge transfer complex can act as a direct source of electrophilic

bromine. In addition, bromine has been shown to react with triethylamine to form a 1:1 charge transfer complex which readily decomposes to N,N-diethylethylideneiminium bromide.[95] Subsequent bromination and aqueous work-up produces N,N-diethyl di- and tribromoacetamides. Furthermore, bromine in chloroform at -10 ^{o}C brominates 1-methylimidazole at the 2,4 and 5 positions.[96] Therefore, if an excess of bromine is used the system is prone to side reactions analogous to those described above thus decreasing the efficiency of the process resulting in laborious purification procedures and therefore reduced yields. Initially, 5'-O-tritylthymidine (15) was used as this meant far simpler ^{1}H n.m.r. spectra and the trityl protecting group is more resistant to removal than is its dimethoxytrityl analogue. In addition, in the preliminary experiments nucleoside residues with thymine as the base were used as they are the least expensive of the four present in DNA and thymidine is usually used without base protection.

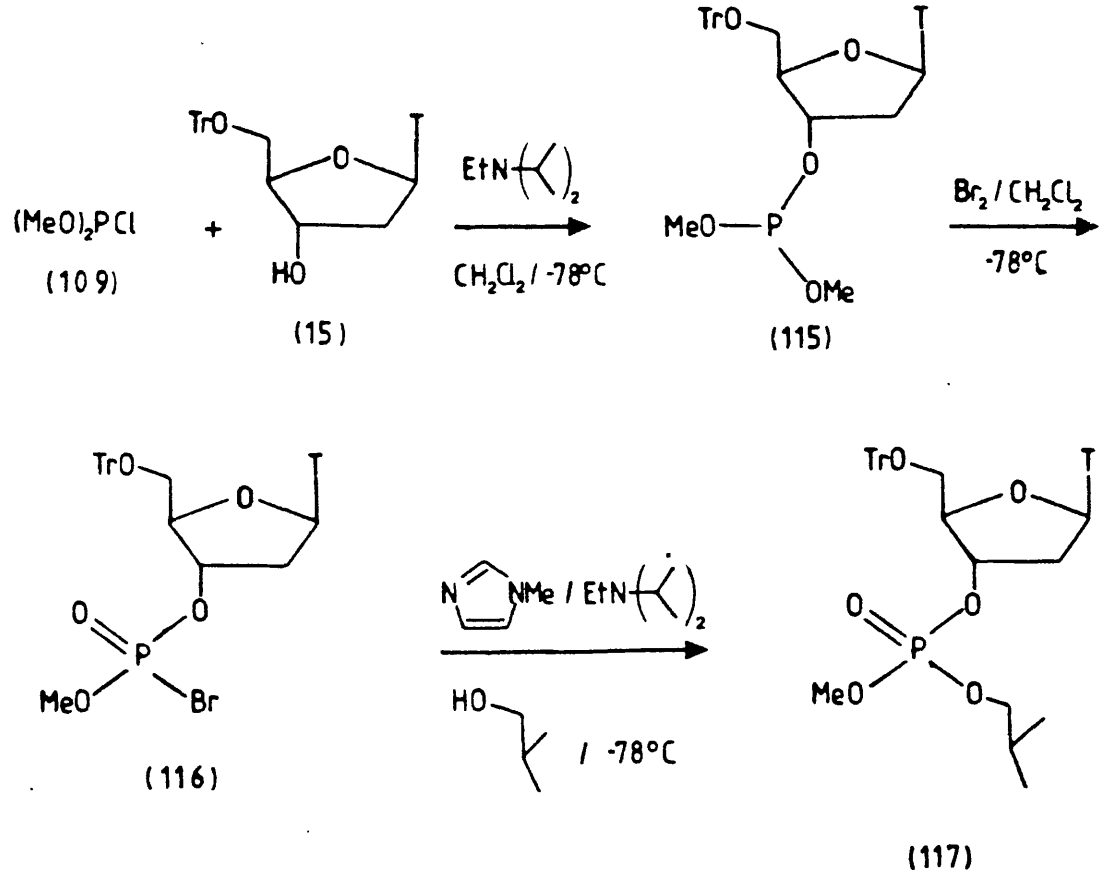
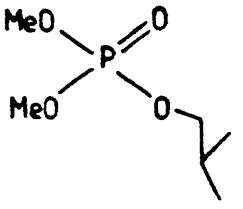


Figure 3.4

Dimethyl phosphorochloridite (109), when treated with 5'-O-tritylthy midine (15) and diisopropylethylamine in methylene chloride at -78 ^{o}C, Figure 3.4, produced a mixture which, in its ^{31}P n.m.r. spectrum, contained a major absorption at +140.3 p.p.m., ascribed to the phosphite (115), and a minor absorption (9% by ^{31}P n.m.r.) at +10.7 p.p.m. due to dimethyl phosphite, resulting from hydrolysis of (109). One molar equivalent of bromine was added. The ^{31}P n.m.r. spectrum showed that the peak at +140.3 p.p.m. had been replaced by two new peaks, of equal intensity, at +4.8 p.p.m. and +4.4 p.p.m., ascribed to the two diastereo-isomers of the phosphorobromidate (116). No absorption was visible at -4.03 p.p.m., characteristic of dimethyl phosphorobromidate (97), suggesting that the nucleoside residue had not been removed in the second stage of the Arbusov reaction. The solution colour had darkened considerably upon the addition of bromine. To this solution, still at low temperature, was added four molar equivalents of 1-methylimidazole (51) and one molar equivalent of isobutanol (111). The ^{31}P n.m.r. spectrum, recorded within five minutes showed a major phosphorus species to be at -0.6 p.p.m. and a minor component at -12.3 p.p.m. (21% by ^{31}P n.m.r.). Following work-up and column chromatography on silica gel the species at -0.6 p.p.m. in the ^{31}P n.m.r. spectrum was isolated in 34% yield and shown to be the required trialkyl phosphate by ^{1}H n.m.r.. The methyl protecting group was shown still to be intact by the presence of two doublets in the ^{1}H n.m.r. spectrum between δ 3.7 and δ 3.9 (J_{P-H}= 11 Hz). The broad absorption at -12.3 p.p.m. in the ^{31}P n.m.r. spectrum is ascribed to pyro-phosphate species which were removed during chromatography.

The product that would have resulted from the formation of dimethyl phosphorobromidate (97) is dimethyl isobutyl phosphate (118), the ^{31}P n.m.r. chemical shift of which is +1.4 p.p.m. This fact was established by carrying out a similar reaction to that described in Figure 3.4,



(118)

δ^{31}P n.m.r (CH$_2$Cl$_2$) +1·4 p.p.m

beginning from trimethyl phosphite and isolating (118) in 40% yield, the major side product being tetramethyl pyrophosphate. However, in the reaction sequence described in Figure 3.4, no absorption at +1.4 p.p.m. was visible throughout. This showed that the reaction had proceeded entirely with loss of methyl bromide in the Arbusov stage to give the required phosphorobromidate (116) with no detectable loss of nucleoside residue to give dimethyl phosphorobromidate (97), and thereby demonstrating the limitations of using the cyclopentyl group as a model for a nucleoside residue.

A possible explanation as to why no loss of the nucleoside unit is observed can be arrived at by consideration of the steric factors involved. The presence of the purine or pyrimidine base residue and the bulky

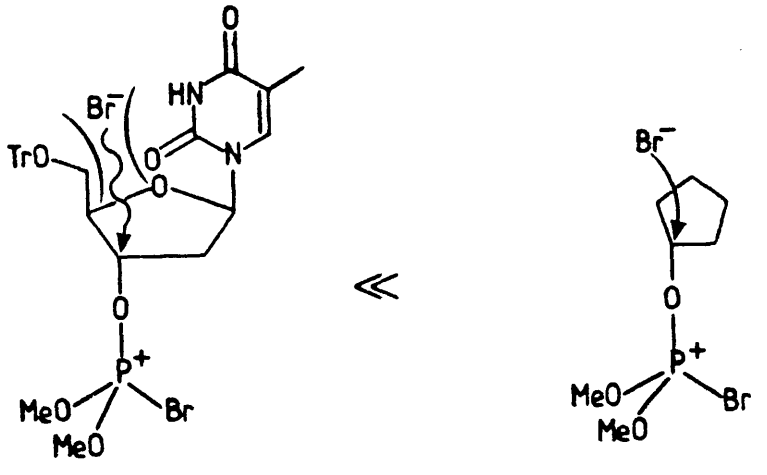


Figure 3.5

trityl substituent would sterically hinder approach of the bromide ion

towards the 3'-position, a situation absent in the cyclopentyl analogue, Figure 3.5. This interference may be so severe that SN2 attack at methyl is preferred.

A similar reaction to that described in Figure 3.4 was carried out using 5'-O-dimeth oxytritylthymidine (10a) and a similar result was obtained, the 'H n.m.r. spectrum of the purified material confirming the presence of the dimethoxytrityl group from the familiar AA' XX' pattern centred at δ 7.1, characteristic of para disubstituted aromatic compounds.

To demonstrate that this procedure could be used to prepare a "natural" dimer, the same reaction sequence was repeated using 3'-O-acetylthymidine (11a) in place of isobutanol, Figure 3.6.

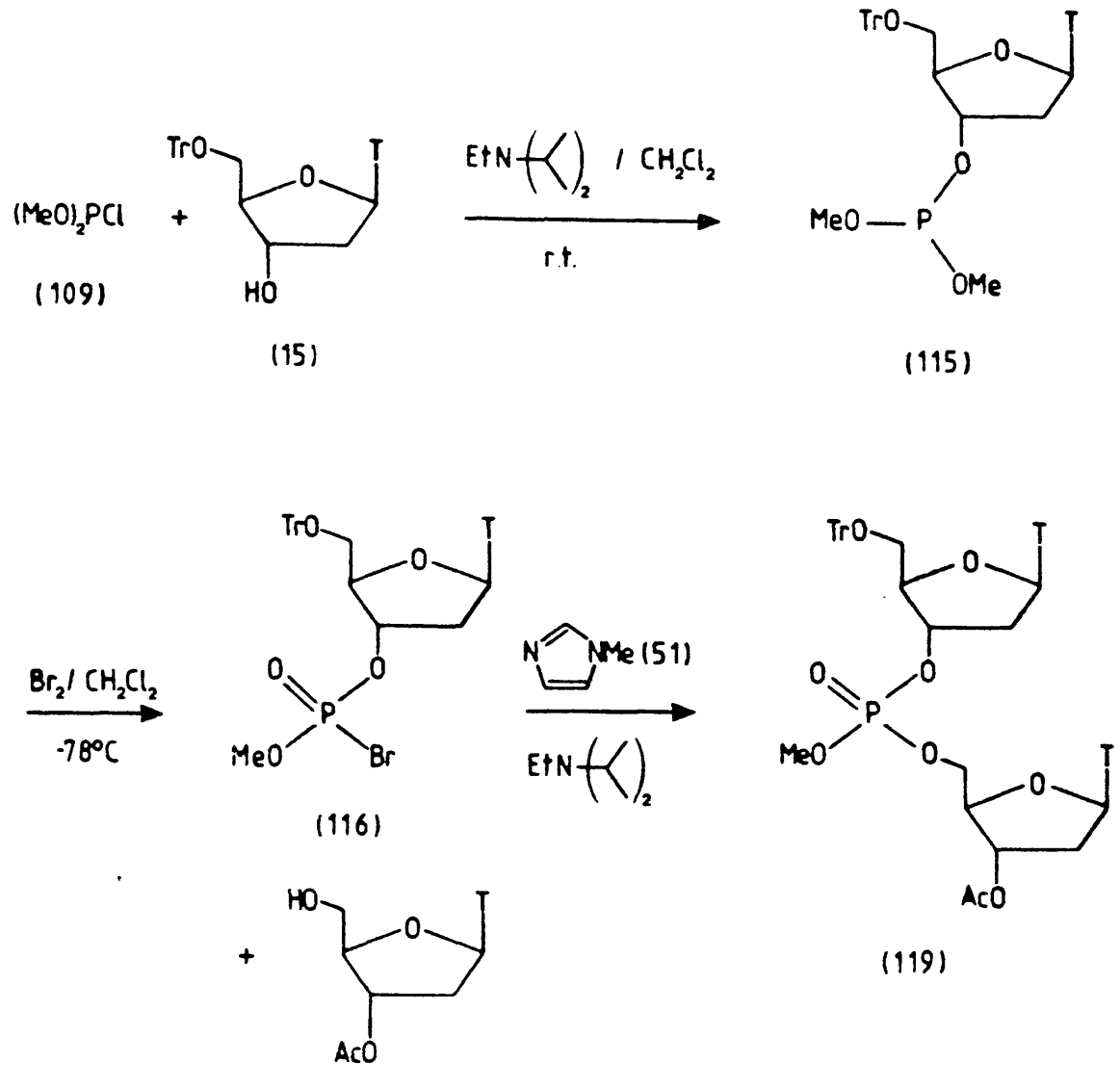


Figure 3.6

The ^{31}P n.m.r. spectrum at the phosphite stage showed a major component having a chemical shift of +140.6 p.p.m., due to (115), and a minor peak at +10.9 p.p.m., due to dimethyl phosphite. The mixture was cooled and one molar equivalent of bromine was added. The ^{31}P n.m.r. spectrum, although it contained the absorption due to (116) as the major component, also contained a significant amount of a material having a chemical shift of -12.3 p.p.m., presumably due to pyrophosphate species. Following the addition of 3'-O-acetylthymidine (11a) and 1-methylimidazole (51), there were three absorptions present in the ^{31}P n.m.r. spectrum at -0.8 p.p.m., -1.4 p.p.m. and -12.5 p.p.m. present as 29%, 20% and 50% (by ^{31}P n.m.r.) of the mixture. Following work-up and removal of the solvent the peak at -12.3 p.p.m. had dramatically decreased in intensity to leave the species at -0.8 p.p.m. as the major component of the mixture and a further peak was present at +1.6 p.p.m. Following column chromatography the species having a chemical shift of -0.8 p.p.m. was isolated in 24% yield and was shown by ^{1}H n.m.r. to be the required fully protected phosphate triester (119). However, in the accepted mechanism of the phosphotriester route an intermediate pyrophosphate species is generated and is subsequently activated by 1-methylimidazole (see Figure 1.20, page 25 and reference 56). If the break down of the pyrophosphate in this experiment occurs by a similar mechanism, Figure 3.7, then during work-up hydrolysis of the 1-methylimidazole-activated phosphate would produce a phosphate diester which may account for the appearance of the new absorption in the ^{31}P n.m.r. spectrum, route (i) in Figure 3.7. However, it is difficult to propose this conclusively without considering route (ii), Figure 3.7, which is how the phosphotriester route had been reported to proceed[56] anyway resulting in formation of the required product.

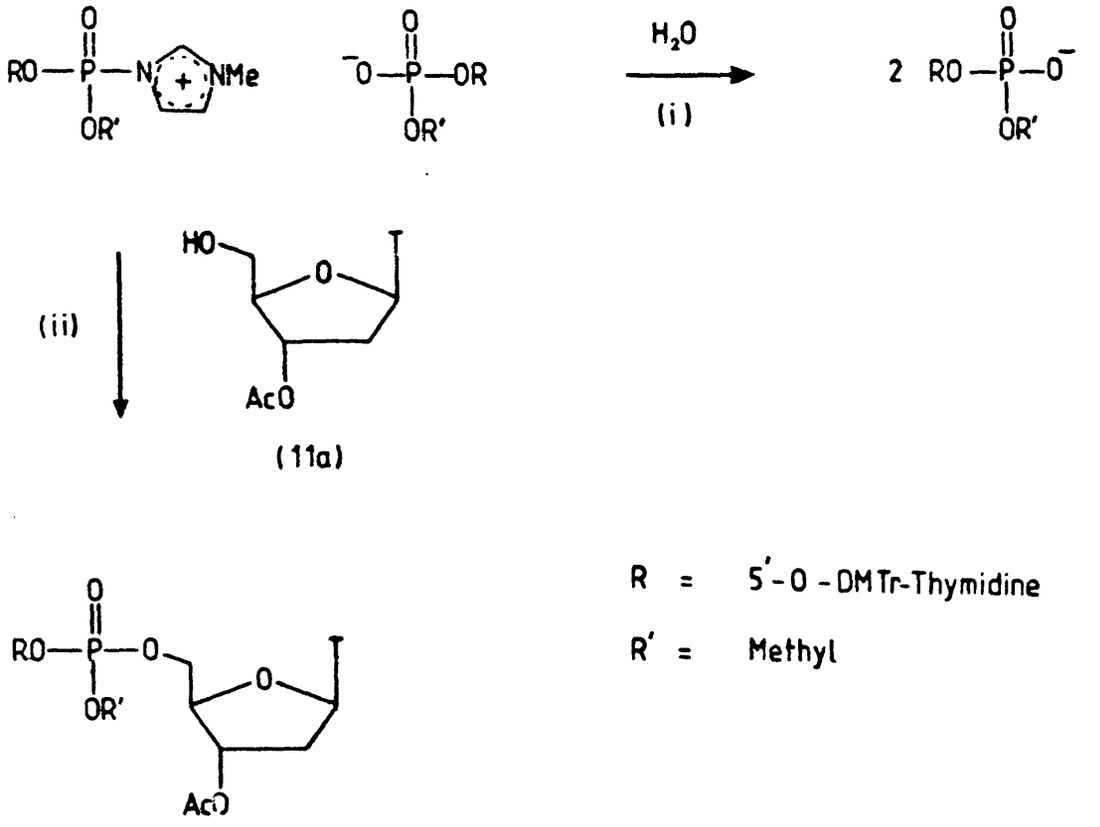


Figure 3.7

Having demonstrated that the dimethoxytrityl group is stable under the conditions of the Arbusov reaction it was now necessary to ensure that the exocyclic amino protecting groups in the bases adenine, guanine and cytosine were stable under the same reaction conditions.

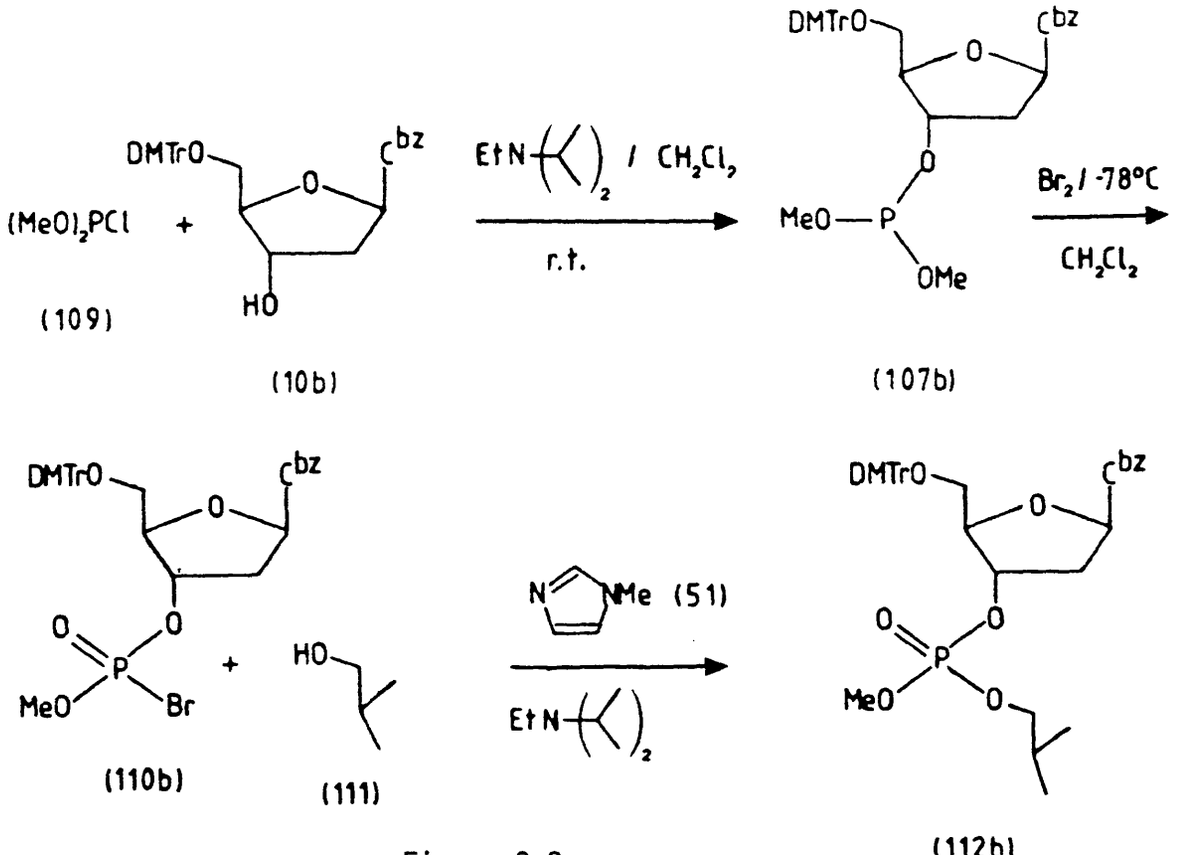


Figure 3.8

As before, after treatment of dimethyl phosphorochloridite with the protected nucleoside residue (10b), Figure 3.8, the ^{31}P n.m.r. spectrum showed the major phosphorus absorption to have a chemical shift of +140.4 p.p.m., due to (107b) and a minor component at +10.9 p.p.m. (11% by ^{31}P n.m.r.). On this occasion the ^{31}P n.m.r. spectrum at the phosphorobromidate (110b) stage was not recorded as, immediately following the addition of one mole equivalent of bromine, isobutanol (111) and an excess of 1-methylimidazole (51) were added. After five minutes the ^{31}P n.m.r. spectrum was recorded. Three major phosphorus absorptions were present at +0.2 p.p.m, -0.8 p.p.m and -1.2 p.p.m present as 14%, 53% and 32% (by ^{31}P n.m.r.) of the mixture respectively. A very minor absorption was present at -12.3 p.p.m. but this was not of significant intensity. The species having the chemical shift of -0.8 p.p.m. was isolated by column chromatography in 25% yield and from integral values in the 'H n.m.r. spectrum it was evident that the benzyl group protecting the amino function attached to the cytosine base had remained intact. It was not clear at this point what the identity of the other components in the reaction mixture were.

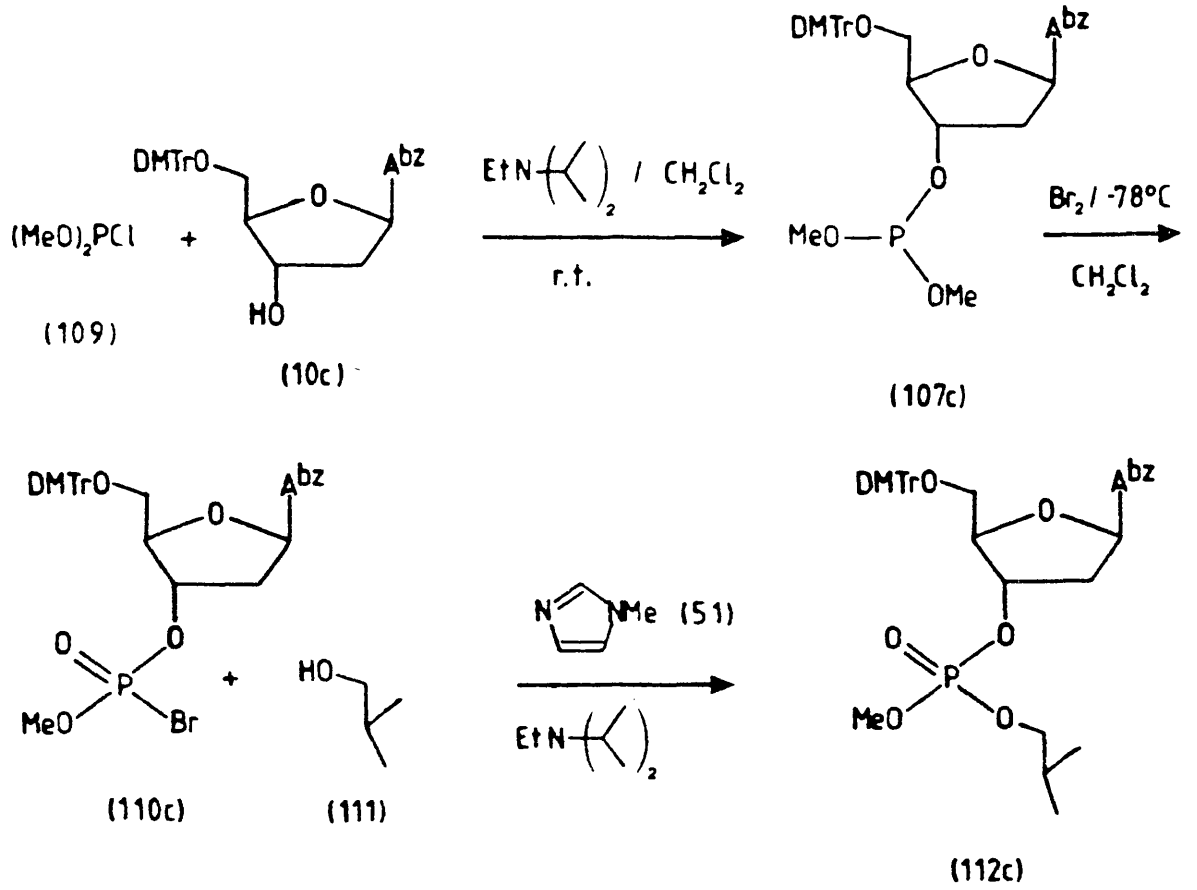
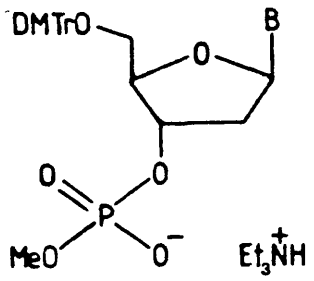


Figure 3.9

The ^{31}P n.m.r. spectrum at the phosphite stage with the protected adenosine derivative showed a major phosphorus absorption at +140.6 p.p.m., due to (107c) and a minor absorption at +10.7 p.p.m. (10% by ^{31}P n.m.r.), again, due to dimethyl phosphite. Following treatment with one molar equivalent of bromine and subsequent treatment with 1-methylimidazole (51, two molar equivalents) and isobutanol (111) in the presence of base produced a mixture the ^{31}P n.m.r. spectrum of which contained two major phosphorus absorptions at +0.4 p.p.m. and -0.8 p.p.m. and a minor absorption at -1.4 p.p.m. present as 21%, 54% and 20% (by ^{31}P n.m.r.) of the reaction mixture respectively. This experimental observation was very similar to that observed in the reaction with the protected cytidine residue. The peak at -1.4 p.p.m., although described as a minor component of the mixture amounts to 26% by ^{31}P n.m.r. as it was very broad. The species at -0.8 p.p.m. was isolated by column chromatography, unfortunately in 17% yield, although it had appeared to be the major component in the crude reaction mixture, and was shown by ^{1}H n.m.r. to be the required phosphate triester (112c).

The polarity of the eluting solvent in the column chromatography procedure was increased and it succeeded in eluting a further compound containing a dimethoxytrityl group and therefore a nucleoside group. This material, by weight, made up 47% of the reaction product and was shown to be the species at +0.4 p.p.m. in the ^{31}P n.m.r. spectrum. The ^{1}H n.m.r. of this compound was consistent with it being the triethylammonium* salt of the phosphate diester, (120c), since it contained a quartet at δ 3.1 and a triplet at δ 1.3 characteristic of the ethyl group. The confirmation

* The triethylammonium salt is obtained as triethylamine was used as a component of the eluting solvent in the chromatography process.



B = T (120a)
B = C^{bz} (120b)
B = A^{bz} (120c)
B = G^{ib} (120d)

(120)

that this is the triethylammonium salt comes from the fact that both of these absorptions were shifted downfield from the corresponding absorptions in triethylamine [δ 2.5 (q) and δ 1.0 (t)]. In addition, no absorption was present at δ 0.9 due to the gem dimethyl groups in the isobutyl substituent and the methyl doublet (J_{P-H} = 10 Hz) was clearly visible at δ 3.5.

The"unidentified" side product in the reaction with the protected cytidine derivative had an identical chemical shift (+0.4 p.p.m.) to that obtained in the previously described experiment. It is therefore reasonable to suggest that this is due to corresponding phosphate diester (120b) formed, presumably from hydrolysis of the phosphorobromidate (110b) although this may have been thought of as being the first stage in the formation of pyrophosphate species, as is suggested in Figure 2.13, page 61.

When the protected guanosine derivative (10d) was subjected to this reaction sequence, Figure 3.10, the ^{31}P n.m.r. spectrum at the phosphite stage contained three phosphorus absorptions at +140.6 p.p.m., +135.5 p.p.m. and +10.5 p.p.m. present as 59%, 16% and 25% (by ^{31}P n.m.r.) of the reaction mixture respectively. The species at +140.6 p.p.m. is assumed, from previous experiments, to be due to the required phosphite (107d) and the absorption at +10.5 p.p.m. is due to dimethyl phosphite resulting from hydrolysis of (109). In addition, and unlike the previous experiments with the three other nucleoside residues, the presence of an extra peak

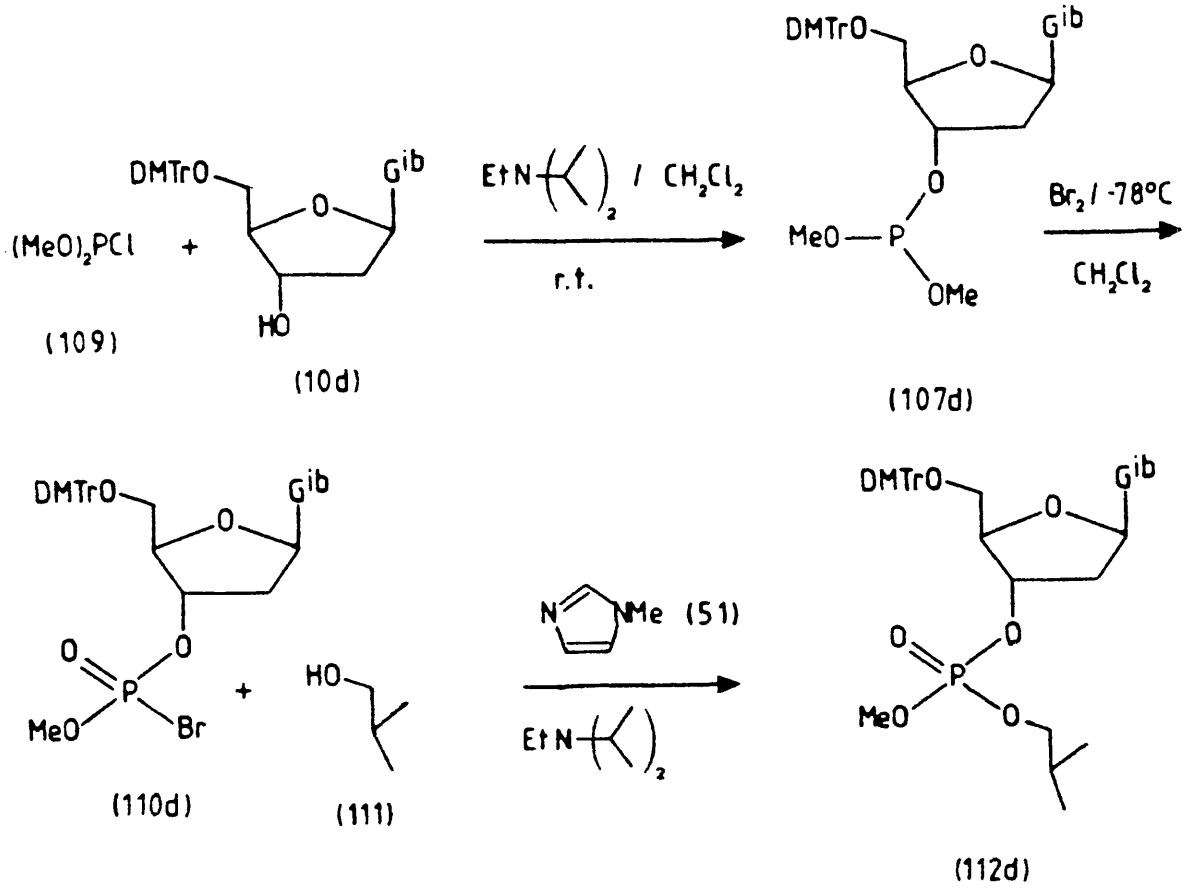


Figure 3.10

(+135.5 p.p.m.) in the phosphite region of the ^{31}P n.m.r. spectrum was observed. In view of the well documented side reactions[10 11 12 13 14] of the O^6 position in the guanine base residue it seems logical to suggest that the species at +135.5 p.p.m. may be due to phosphitylation at the O^6 position. Although, when side reactions at the O^6 position occur, some of the derivatives formed may be converted back to the parent compound,[9798] in many instances they have been reported to degrade to polar, fluroescent materials.[12] Even with this consideration it was not predictable as to what the outcome of this sequence of reactions on a species phosphitylated at the O^6 position would be.

Following the addition of bromine and subsequent addition of isobutanol and 1-methylimidazole in the presence of base, the ^{31}P n.m.r. spectrum displayed two major phosphorus absorptions at +0.6 p.p,m. and

-0.4 p.p.m. present as 37% and 63% (by ^{31}P n.m.r.) of the mixture respectively. Minor absorptions were present at -7.1 p.p.m. and at +10.9 p.p.m. due to the unchanged dimethyl phosphite. The initial chromatographic purification of the crude product did not succeed in separating the species at -0.4 p.p.m. completely from other dimethoxytrityl containing material (by tlc), this being effected by preparative thin layer chromatography to give a 20% yield of this material which was shown by ^{1}H n.m.r. to be the required phosphate triester.

The confirmation that the amino protecting group had remained intact was evident from the presence of two overlapping doublets (J = 7 Hz) centred at δ 1.1. due to the diastereotopic methyl groups in the isobutyryl side chain.

The presence in the ^{31}P n.m.r. spectrum of the crude material of a species at +0.6 p.p.m. seemed to indicate that a considerable amount of the phosphate diester (120d) had also been formed. This material was eluted from the column by increasing the polarity of the eluting solvent in addition to several other dimethoxytrityl containing species (by tlc). The ^{31}P n.m.r. spectrum of this mixture displayed several phosphorus absorptions at +1.4 p.p.m., +0.4 p.p.m., -0.4 p.p.m. and -7.1 p.p.m. present as 36%, 24%, 27% and 12% (by ^{31}P n.m.r.) of the mixture respectively. The identity of three of these materials is not known but are speculated to be due to side reactions involving the O^6 position of the guanine residue as, in addition to the suggested phosphitylation at the O^6 position Reese[10] has shown that the O^6 position can be phosphorylated also and indeed, uses this reaction en route to the protection of the O^6 position.

The poor yields obtained could be due to several factors.

(i) Possible competition in the phosphorylation step from the 5'-O-protected monomer, which will be present in excess if a significant amount of the dimethyl phosphorochloridite (109)

has been consumed by hydrolysis.

(ii) The formation of pyrophosphate species, possibly due to trace amounts of water present in the reagents was observed, which then may break down to the phosphate diesters observed in some cases. Alternatively, the phosphate diesters (120) may be obtained by direct hydrolysis of the phosphorobromidate (110) and not via the pyrophosphate as, although 1-methylimidazole has been reported to attack pyrophosphate species[56] (see Figure 1.20, page 25), the pyrophosphate species obtained appear to be stable in the presence of 1-methylimidazole under the conditions used. An alternative mechanism to pyrophosphate species has been reported by Simpson and Zwierzak,[99] Figure 3.11.

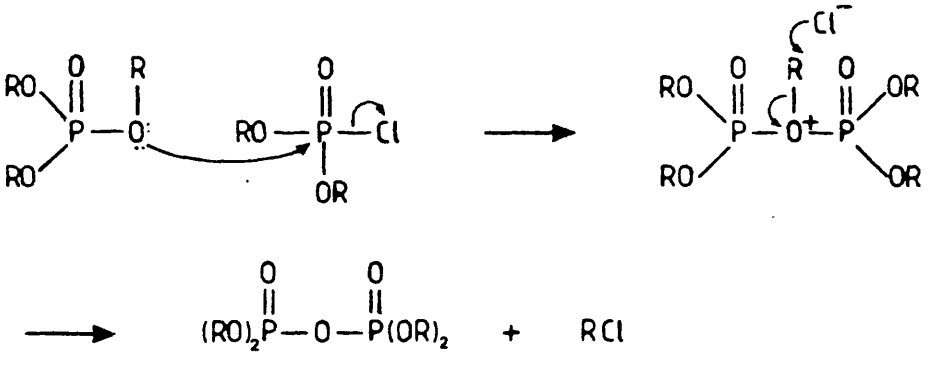


Figure 3.11

The mechanism has been reported to proceed by nucleophilic attack of the alkoxy oxygen of a trialkyl phosphate on a phosphorochloridate displacing Cl^-, which then dealkylates the bridging oxygen to give the pyrophosphate. This, however, may not be directly relevant to the phosphorylation reactions undertaken here, since the conditions for the two are dissimilar, the Simpson and Zwierzak experiments being conducted in refluxing xylene (b.pt 144 ^oC) over a two hour period.

(iii) In the case of the guanine residue, products derived from side reactions at the O^6 position result in decreased yields.

Due to the number of reagents used in the reaction sequences described and the potential side reactions that can occur the stoichiometries need to be measured carefully. In addition, the phosphorus intermediates throughout the sequence are extremely susceptible to hydrolysis and as the nucleoside units used are very hygroscopic a substantial amount of experience is required before satisfactory repeatable yields are obtained. Under optimum conditions, therefore, isolated yields up to 69% can be

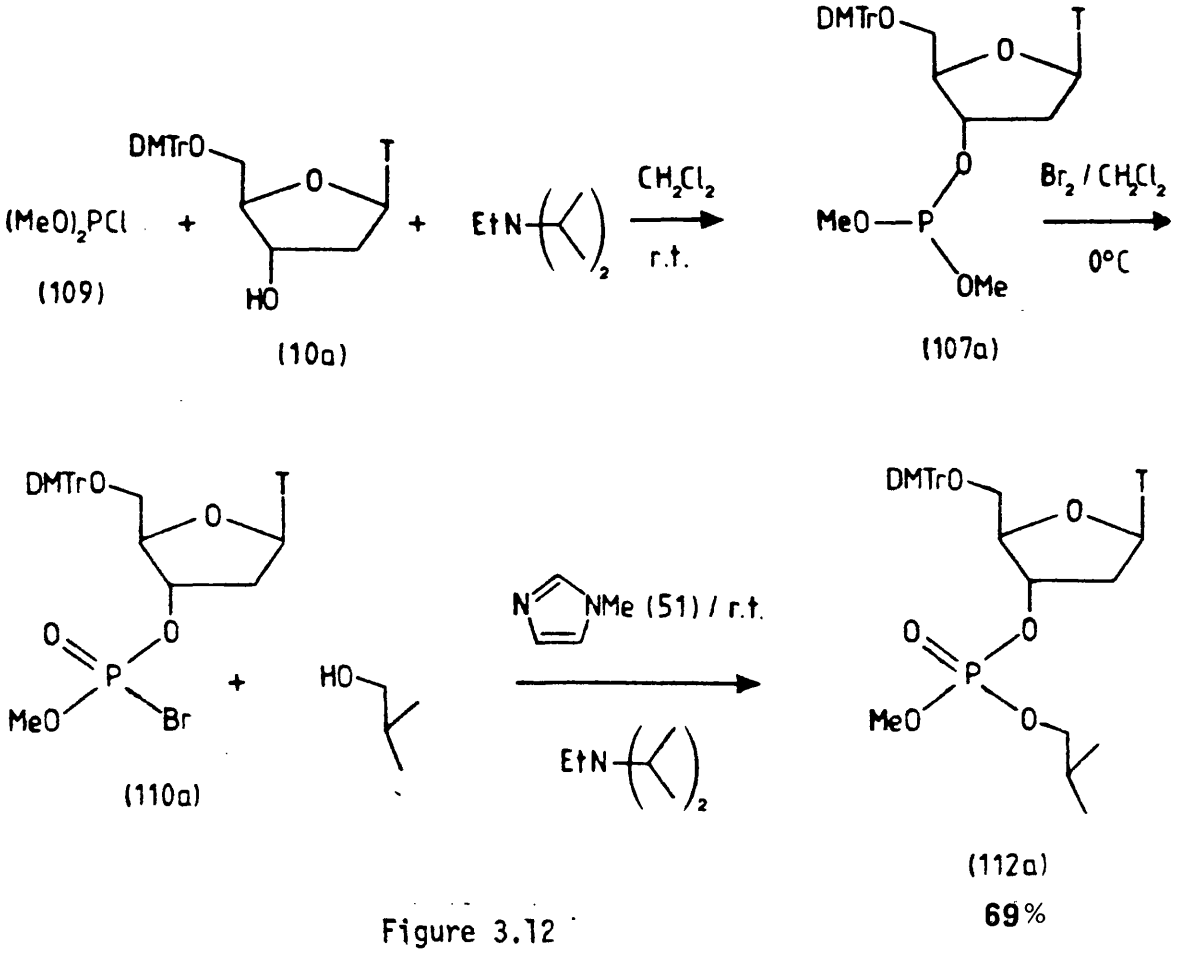


Figure 3.12

obtained, Figure 3.12, with one molar equivalent of 1-methylimidazole being used. Figure 3.13 shows a set of ^{31}P n.m.r. spectra recorded at various stages in the above reaction sequence. The spectra are normally recorded on a 10 KHz sweep width. This was not sufficient, however, to

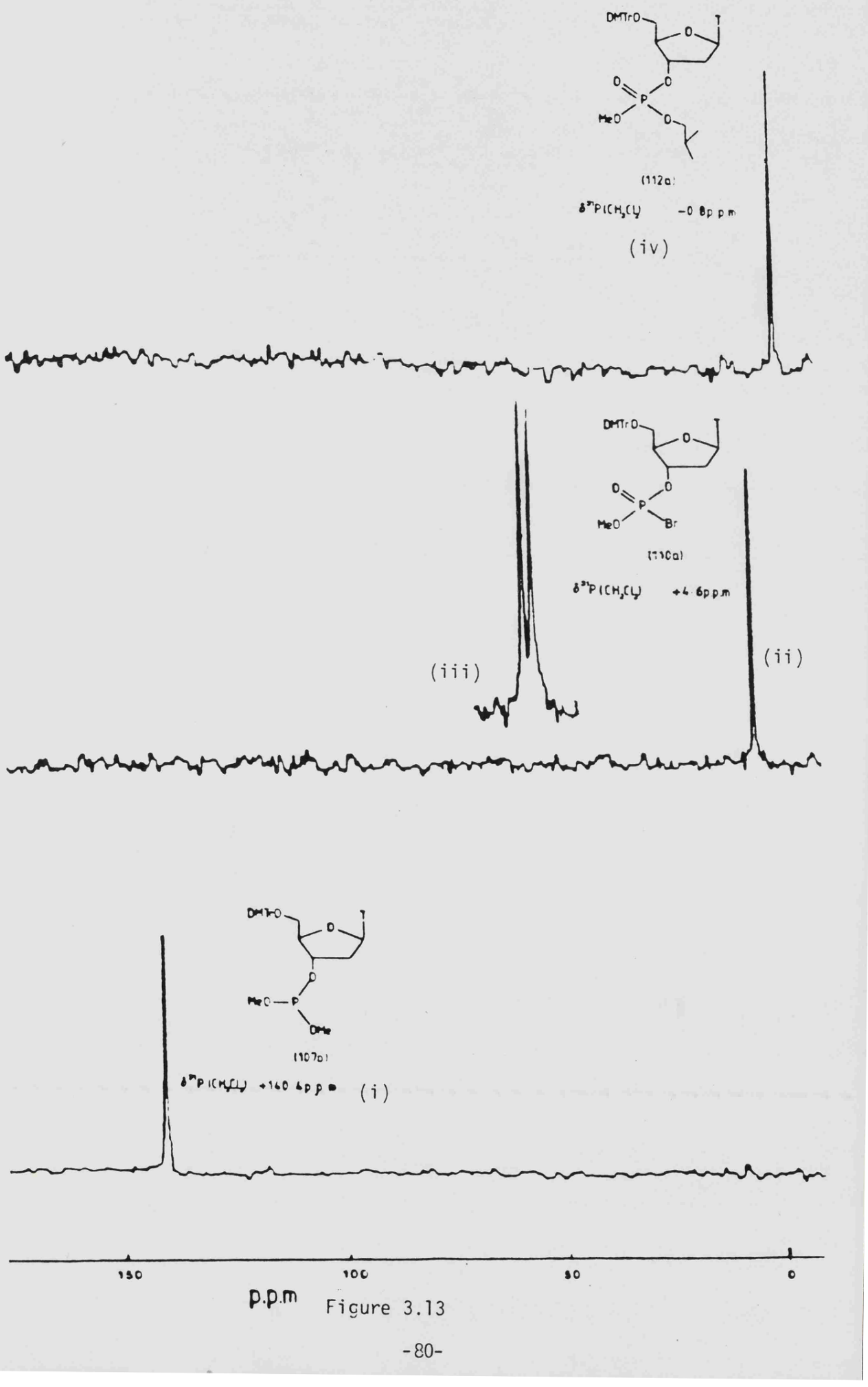

DMTrO
MeO
(112a)
$\delta^{31}P(CH_2Cl_2)$ −0.8 p.p.m
(iv)
DMTrO
MeO
Br
(110a)
$\delta^{31}P(CH_2Cl_2)$ +4.6 p.p.m
(iii)
(ii)
DMTrO
MeO
OMe
(107a)
$\delta^{31}P(CH_2Cl_2)$ +140.4 p.p.m
(i)
150
100
50
0
p.p.m

Figure 3.13

resolve the two disatereoisomers of the phosphorobromidate (110a), this condition being effected by re-recording Figure 3.13 (ii) on a 1 KHz sweep width, Figure 3.13 (iii).

It was not anticipated prior to these experiments and indeed, was subsequently not observed, that the presence of bromine would result in modification of the purine and pyrimidine bases in any way as halogenation of the nucleosidic bases requires rather different conditions and substantially longer reaction times than those employed here. For example, 2'-deoxyadenosine can be brominated at position 8 by treatment with bromine water in 0.1 N sodium hydroxide.[100] This reaction requires 96 hours however, to give a 66% yield of 8-bromo-2'-deoxyadenosine. The reaction of tetrabutylammonium iodotetrachloride with 2'deoxyadenosine in DMF gives the same product in 38% yield,[101] the reaction time in this case being 24 hours. Uridine has been brominated at position 5 using (bromomethylene) dimethylene ammonium bromide although reaction conditions of 120 ^{o}C for 8 hours are required.[102]

In the course of optimisation of the yields, apparent hydrolysis of intermediates had been the major cause of side reactions. This was not considered to be of undue concern with regard to solid phase synthesis as an excess of the phosphorylating agent is used. Therefore, even if a substantial amount of material had been lost due to side reactions it was still anticipated that sufficient phosphorylating agent would be present to react with the 5'-hydroxyl component attached to the polymer support. In addition the side products obtained were not expected to react with the 5'-hydroxyl component. Thus, they would be easily removed by washing.

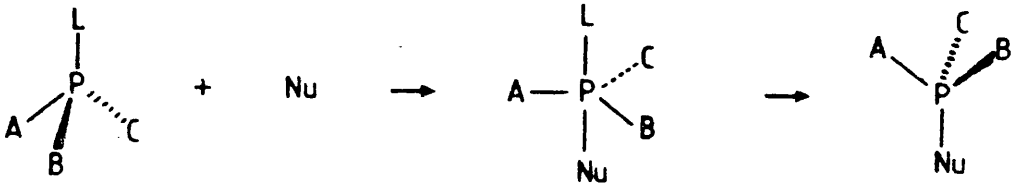
The next stage towards the synthesis of oligonucleotides was to apply the methodology developed in solution to solid phase synthesis. This is described in Chapter 6.

CHAPTER 4

THE REACTION OF PHOSPHOROBROMIDATES WITH ALCOHOLS IN THE PRESENCE OF VARIOUS ACTIVATING AGENTS

The Reactions of Phosphorobromidates with Alcohols in the presence of various Activating Agents

Criterion (iii) (page 43) concerns the reactivity of phosphoryl halides, of the type generated in the Arbusov reaction, with alcohols, the next stage in the formation of the phosphate triester. Nucleophilic displacement reactions at tetracoordinate pentavalent phosphoryl centres can often occur with high stereoselectivity with either essentially complete inversion or retention of configu ration. Reactions that occur with inversion of configuration are customarily considered to occur by an SN2 (P) mechanism through a trigonal bipyramidal (TBP) intermediate, Figure 4.1, with attack of the nucleophile (Nu), being opposite to that of the leaving group (L). The nucleophile and the leaving group are



(TBP)

Figure 4.1

therefore immediately in apical positions in the TBP. Therefore, subsequent departure of the leaving group (L) before any ligand reorganisation can take place results in inversion of configuration.

The concept of a TBP intermediate permits an explanation[103 104 105] for nucleophilic displacements at phosphorus that occur with retention of configuration. Attack at phosphorus occurs opposite to a ligand that is not a leaving group, Figure 4.2. The leaving group (L) takes up the apical position from which it departs following ligand reorganisation via a pseudorotation process.[106 107]

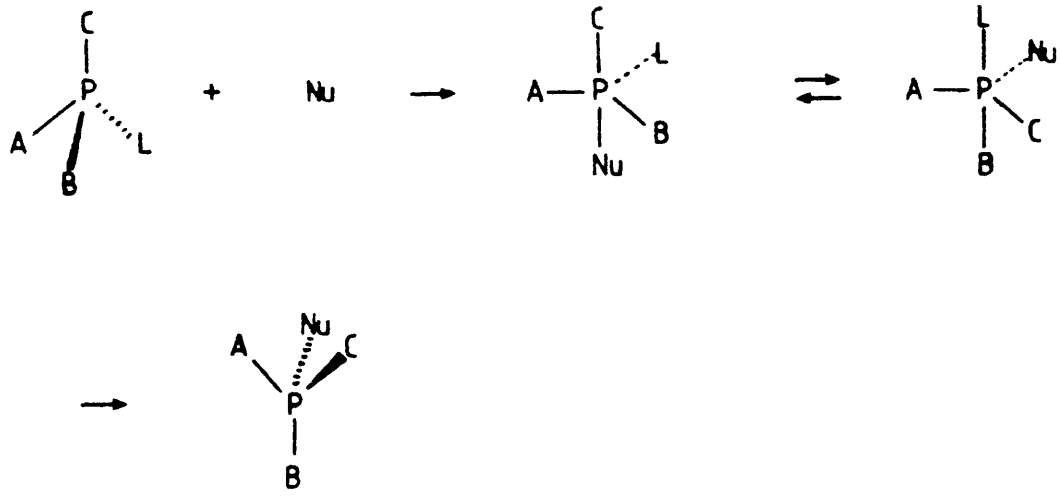
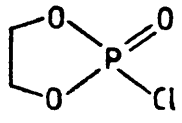
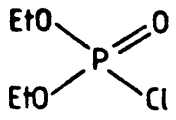


Figure 4.2

Where phosphorus is part of a five-membered ring Westheimer[108] noted a large increase in the rate of exocyclic hydrolysis of methyl ethylene phosphate compared to the hydrolysis of trimethyl phosphate ($\sim 10^7$) this fact being partly explained by the relief of ringstrain in going to TBP intermediate. However, Corriu[109] has not observed a similar rate relation in the hydrolysis of the phosphorochloridates (121) and (122), the relative rate being 3×10^2. Furthermore,in a series of reactions



(121)



(122)

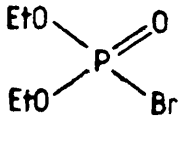
$$k_{121} / k_{122} = 3 \times 10^2$$

carried out where (121) and (122) are used as phosphorylating agents for various nucleophiles, (121) is found to phosphorylate in all cases more rapidly than (122), the difference being dependent upon the nucleophile. The most relevant reaction from this series is the formation of a phosphate

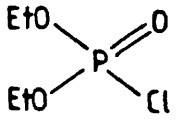
triester by reaction of (121) and (122) with $EtOH/Et_3N$, the relative rate being $K(121)/K(122) = 9 \times 10^4$.

With respect to oligonucleotide synthesis this poor reactivity of phosphorochloridates where phosphorus is not part of a ring can be put into perspective when considering the initial experiments of Reese[35] (Figure 1.12, page 17). Using phenyl phosphorodichloridate (35), two phosphorylations are required. The first, with a 5'-O-protected nucleoside in the presence of 2,6-lutidine, carried out at 20 ^{o}C in anhydrous acetonitrile requires 36 hours, the second, under the same conditions but using a 3'-protected monomer, requires 48 hours. Similarly, Eckstein[32], (Figure 1.11, page 15), using 2,2,2-trichloroethyl phosphorodichloridate (31) with a 5'-protected monomer with 2.5 molar equivalents of pyridine in chloroform at room temperature required 24 hours for the first phosphorylation. Following evaporation, the second phosphorylation was carried out in pyridine as solvent with a 3'-protected monomer at room temperature over a 48 hour period. The second phosphorylation in both of these cases bears a close resemblance to our proposed Arbusov reaction route and for this to be a viable alternative to the existing methodologies conditions needed to be found whereby the phosphoryl halides produced following the Arbusov reaction effected rapid phosphorylation of an alcohol, a 48 hour reaction time clearly being unacceptable.

Diethyl phosphorobromidate (123) has been reported as being a superior phosphorylating agent to the corresponding chloridate (122) in peptide synthesis.[75] Therefore, it would seem logical to suggest that



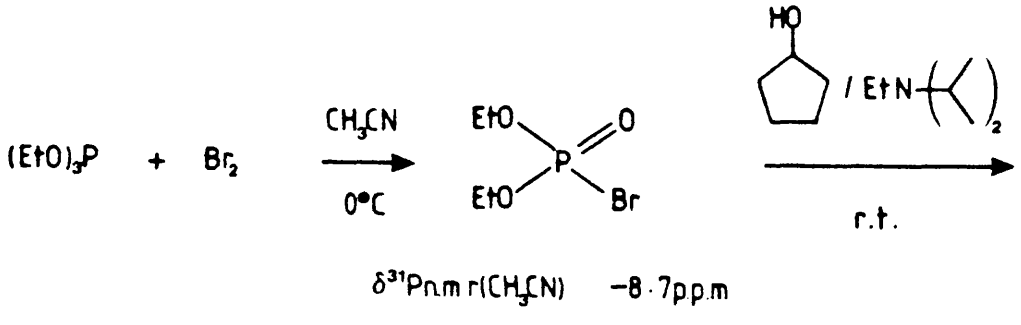
(123)



(122)

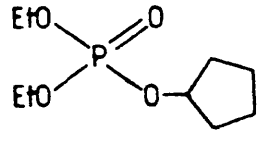
phosphorobromidates would be more suitable in the proposed synthetic route to oligonucleotides involving the Arbusov reaction, with the added advantage that elemental bromine, experimentally, is easier to handle than elemental chlorine (the reason why Arbusov reactions carried out in preceding chapters were conducted using bromine).

Initially, model experiments were carried out on diethyl phosphorobromidate (123) as the phosphorylating agent.



δ^{31}P n.m.r.(CH_3CN) −8.7 p.p.m

(123)



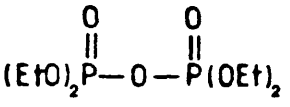
(124)

δ^{31}P n.m.r.(CH_2Cl_2) −2.2 p.p.m

Figure 4.3

Triethyl phosphite was treated with one molar equivalent of bromine at 0 ^{o}C in acetonitrile solution, Figure 4.3. The reaction is exothermic. The ^{31}P n.m.r. spectrum at this point showed that the signal due to triethyl phosphite (+138.4 p.p.m.) had been replaced by a single broad peak at -8.7 p.p.m. due to (123).[75] To the solution, after having been warmed to room temperature, was added two molar equivalents of diisopropylethylamine and one molar equivalent of cyclopentanol, the subsequent reaction being monitored by ^{31}P n.m.r. spectroscopy. After 45 minutes, the major

phosphorus absorption in the ^{31}P n.m.r. spectrum was still that due to (123) although two further signals were visible as minor components of the mixture at -2.2 p.p.m. and -13.3 p.p.m., both of comparable intensity. The reaction took a further 16 hours for the signal due to (123) to disappear and to be replaced by the signal at -2.2 p.p.m. as the major species present (66% by ^{31}P n.m.r.) and the signal at -13.3 p.p.m., present as 34% of the reaction mixture. Following work-up and vacuum distillation, the species at -2.2 p.p.m. was isolated in 40% yield and was shown by ^{1}H n.m.r., I.R and mass spectral data to be the required product, cyclopentyl diethyl phosphate (124). The species at -13.3 p.p.m. is tetraethyl pyrophosphate (125).[110]

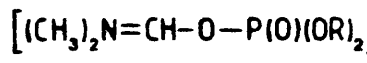


(125)

By comparison with the second phosphorylation step in the Reese[35] and Eckstein[32] phosphorochloridate reactions, diethyl phosphorobromidate does phosphorylate more effectively although a reaction time of 16 hours is still unacceptable for an efficient synthesis.

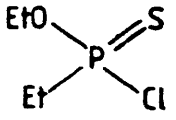
DMF as an Activating Agent

DMF has been reported to catalyse the reactions of phosphorochloridates with nucleophilic reagents (water, alcohols, amines and acids), the acceleration commonly being explained in terms of the formation of a reactive DMF-phosphorus adduct, postulated for dialkyl phosphorochloridates to be structure (126).[111]



(126)

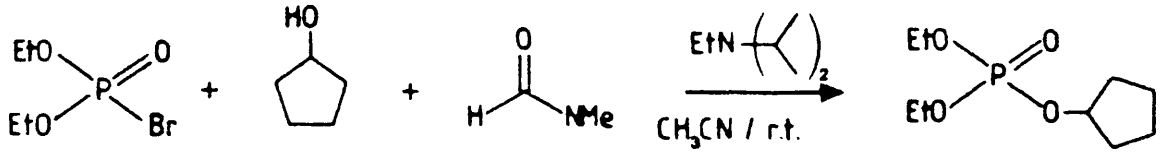
Mikolajczyk,[112] in the hydrolysis of optically active O-ethyl ethylphosphorochloridothionate (127) in DMF/water mixtures discounted the



(127)

intermediacy of a structure analogous to (126) as overall inversion of configuration was observed when overall retention was expected from two inversions at the thiophosphoryl centre. The reaction, however, is reported to proceed more rapidly than without the presence of DMF, this being attributed to the higher polarity and better solvating properties of the reaction medium. More recently,Corriu [113] has reported nucleophilic participation of DMF in the hydrolysis of chlorophosphonates.

It was therefore envisaged that DMF might effect a similar rate enhancement in the reaction of diethyl phosphorobromidate (123) with cyclopentanol. Therefore, (123) was generated by treatment of triethyl



(123) (124)

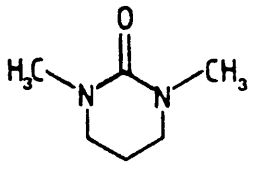
phosphite with bromine. Two molar equivalents of DMF were added and the ^{31}P n.m.r. spectrum recorded before the addition of cyclopentanol indicated no change in the chemical shift of (123) from -8.6 p.p.m., although a small amount of (125) was present at -13.5 p.p.m. This, therefore, tends to refute the formation of a phosphorus-DMF adduct analogous to (126), although it may be an equilibrium process which at room temperature is over to the side of (123). Addition of one molar equivalent of cyclo-

pentanol resulted in rapid disappearance of the signal due to (123) and the appearance of two signals of comparable intensity (52% and 48% by ^{31}P n.m.r.) at -2.2 p.p.m. and -13.5 p.p.m. The species at -2.2 p.p.m. was shown to be the required phosphate (124), it being identical to that prepared in the uncatalysed process. The pyrophosphate (125) is assumed to arise from trace amounts of water believed to be present in the DMF although the DMF had been "dried" using the method of Thomas and Rochow[114], it having been benzene azeotroped, shaken with powdered barium oxide, decanted (after standing over barium oxide overnight) and distilled under reduced pressure.

The presence of basic materials catalyses the decomposition of DMF to dimethylamine and carbon monoxide although no diethyl N,N'-dimethyl-phosphoramidate (δ ^{31}P +11.1 p.p.m.) was observed in the ^{31}P n.m.r. spectrum. In addition, this report[114] recognises that trace amounts of water are always present in DMF regardless of the method chosen for its purification. This suggests that DMF is unsuitable for rate enhancement in this phosphorylation process, especially in view of the apparent ease of formation of tetraethyl pyrophosphate from diethyl phosphorobromidate.

DMPU (128) as an Activating Agent

The dipolar aprotic solvent N,N'-dimethyl-N,N'-propylene urea (DMPU, 128) has been used[115] as an alternative to the carcinogenic



DMPU (128)

hexamethylphosphoric triamide as a cosolvent in reactions requiring the presence of a dipolar aprotic solvent. Recently,[116] Seebach has used (128) as a cosolvent in diastereoselective alkylation of oxazolines to yield 4,4-disubstituted oxazolines. The resemblance that DMPU bears to DMF makes it a potential reagent for use in phosphorylation reactions with a view to rate enhancements. In addition, although it is hygroscopic it can be dried by distillation from calcium hydride under reduced pressure; an obvious advantage over DMF.

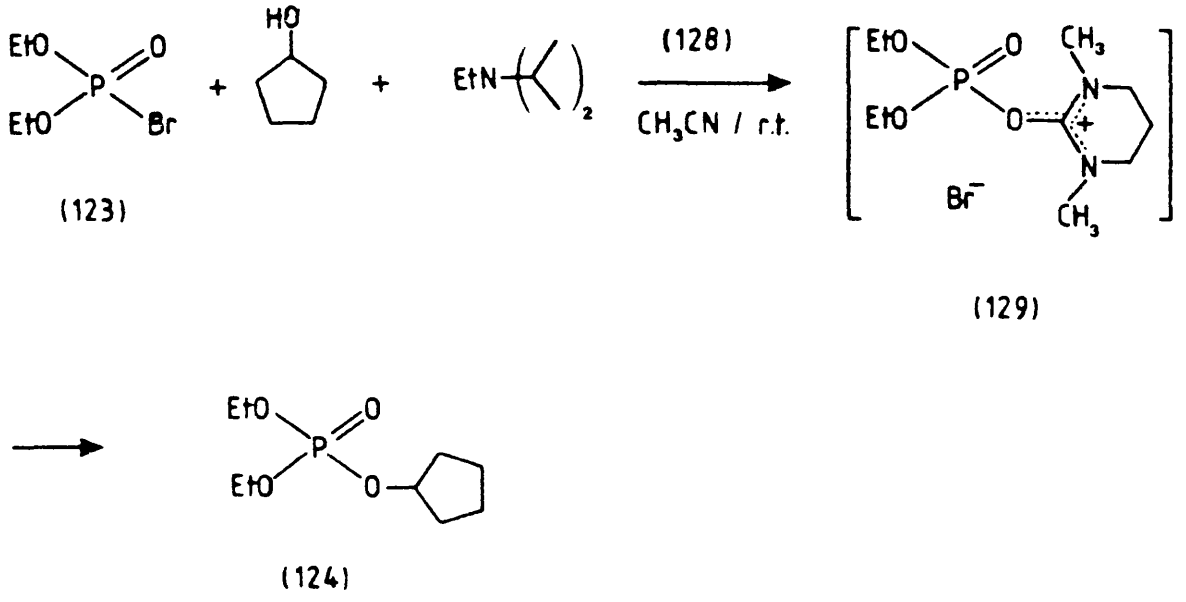


Figure 4.4

Diethyl phosphorobromidate (123) was generated as before and to a solution of this in acetonitrile at room temperature was added one mole equivalent of cyclopentanol and 1.1 molar equivalents of DMPU in the presence of base. The subsequent reaction was monitored by ^{31}P n.m.r. and a set of spectra describing the progress of the reaction at various times from five minutes to four hours are shown in Figure 4.5.

Interestingly, the absorption due to diethyl phosphorobromidate (123) is observed to decrease in intensity in accordance with the emergence of a new absorption to the high field side of the starting material at -9.9 p.p.m. The intensity of this absorption appears to

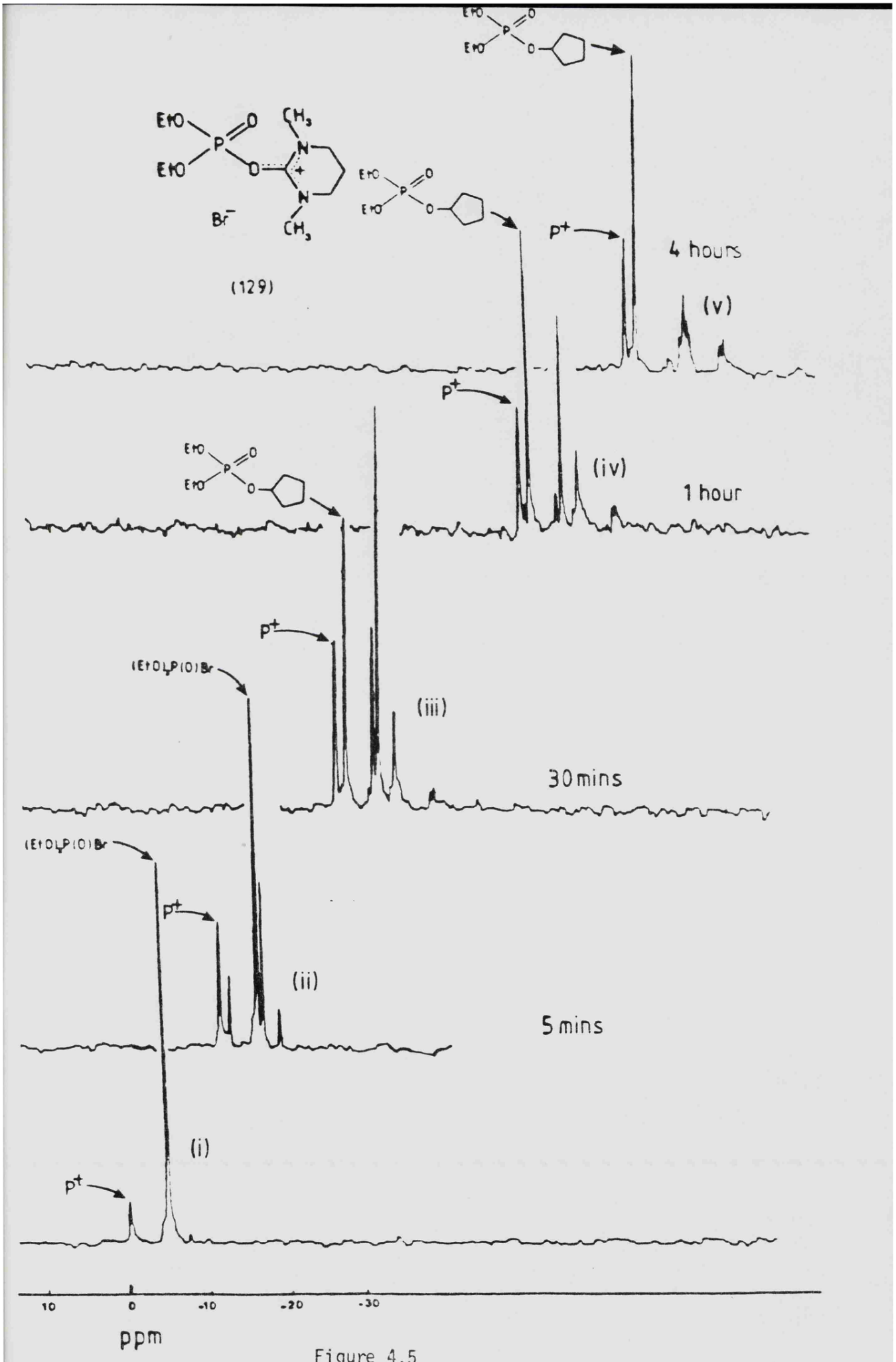
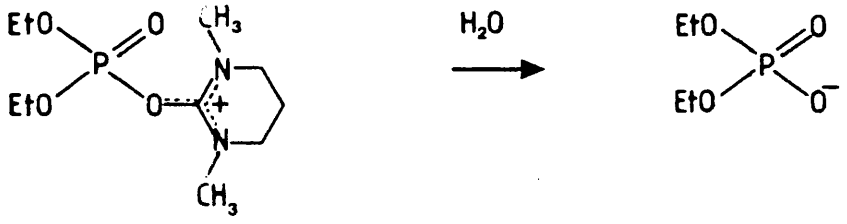

(129)
4 hours
(v)
1 hour
(iv)
30 mins
(iii)
5 mins
(ii)
(i)
p+
(Et0)2P(0)Br
10
0
-10
-20
-30
ppm

Figure 4.5

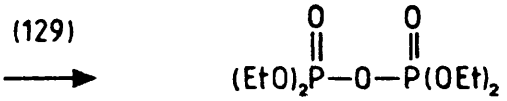
reach a maximum after approximately 30 minutes, Figure 4.5 (iii), and then, over the next three hours, slowly decreases in intensity in accordance with the emergence of an absorption at -2.2 p.p.m., later shown to be due to the required trialkyl phosphate (124), Figures 4.5 (iii), (iv), (v). In addition to the peak due to the required product, as the peak due to the apparent intermediate collapses, it also gives rise to a cluster of peaks between -12.7 p.p.m. and -15.1 p.p.m., in the pyrophosphate region of the spectrum and at -20 and -22.9 p.p.m.

Species having chemical shifts of ~-20 p.p.m. in the ^{31}P n.m.r. spectrum are normally associated with trimetaphosphate type species, and indeed inorganic trimetaphosphate has a chemical shift of -20.7 p.p.m.[117]

A trimetaphosphate species (21), page 10 , is reputed to be an intermediate in the phosphodiester route[27] and is speculated to arise from monomeric metaphosphate formation, Figure 1.7, page 11. Although the formation of tetraethyl pyrophosphate may be thought of as arising



(129)



(125)

Figure 4.6

through hydrolysis of the speculated intermediate (129), the acid obtained reacting with a further equivalent of (129) to give (125), Figure 4.6, several other pyrophosphate species are present, which may be alkylated

with ethyl and cyclopentyl groups or may not be fully alkylated at all.

A possible explanation as to how several pyrophosphate type species, and trimetaphosphate species are formed is outlined in Figure 4.7 via the monomeric metaphosphate (130). A series of reactions such as those shown in Figure 4.7 would account for the several byproducts obtained in addition to the required trialkyl phosphate although there is no direct experimental evidence to confirm that reactions of this kind are actually occurring.

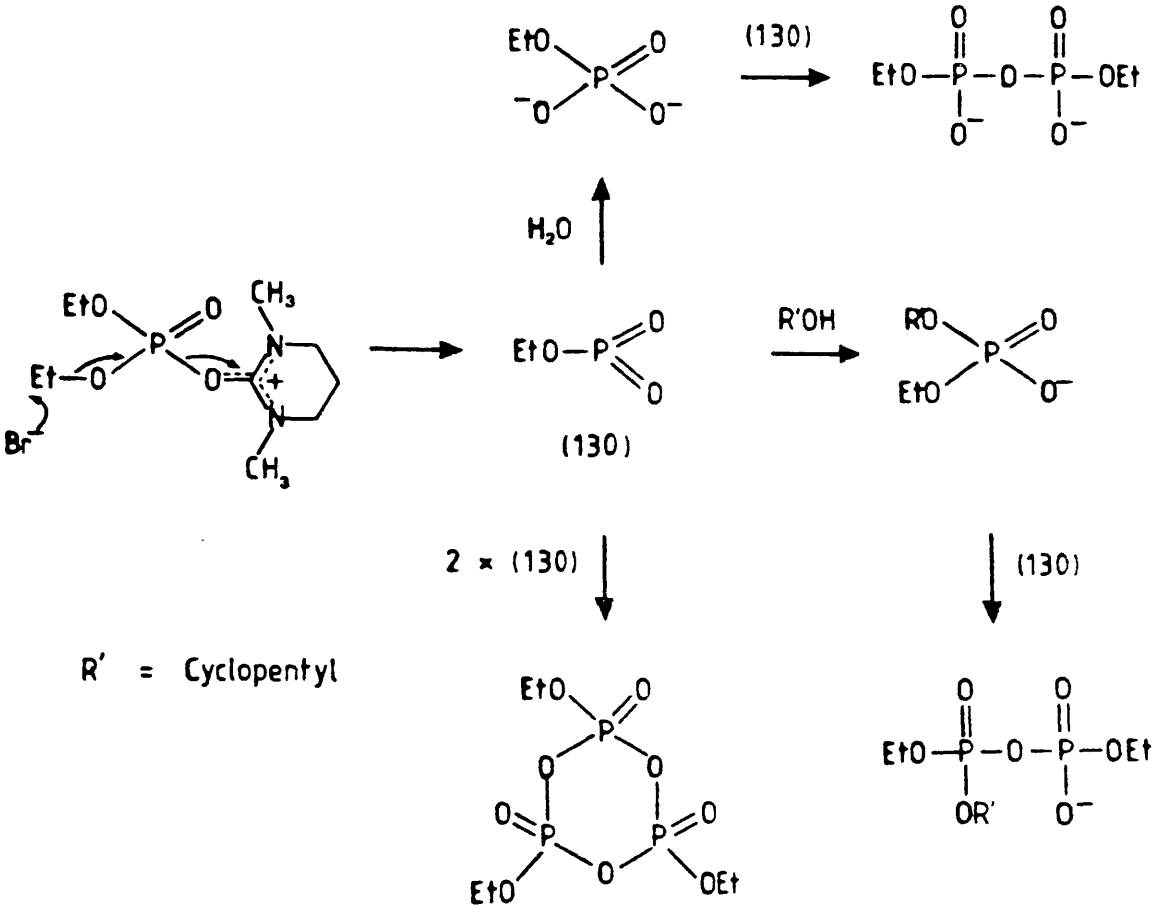


Figure 4.7

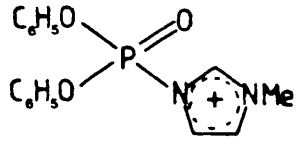
As several side products were observed, the required product (124) was only isolated in 25% yield.

Although this experiment aroused a certain amount of interest with regard to a rapid phosphorylation process a reaction time of four hours was still unacceptable. A subsequent experiment where a greater excess of DMPU was used (five molar equivalents) reduced the reaction time to one hour although this was still considered to be too slow.

1-Methylimidazole (51) as an Activating Agent

Tetrazole (72), in the presence of arylsulphonyl chlorides has been shown[52] to produce an efficient phosphorylation procedure in the phosphotriester route and although the phosphorylation reactions of Eckstein[32] using pyridine as the base require 24 hours and 48 hours respectively, pyridine has been reported as behaving as a nucleophilic catalyst.[57] Initial experiments using tetrazole and pyridine were encouraging but they were not thoroughly investigated as the rapid phosphorylation of an alcohol was achieved with 1-methylimidazole (51) as activating agent.

The idea of using 1-methylimidazole as a nucleophilic catalyst is not new. Westheimer has shown[118] that in the solvolysis of tetrabenzyl pyrophosphate in the presence of (51), catalysis is effected by nucleophilic attack of (51) at phosphorus forming the N-(dibenzylphosphoryl)-1-methylimidazolium ion (131).



(131)

A dianion species analogous to (131) has been reported[119] as an intermediate in the phosphorylation of amines in aqueous solution and Takaku has reported the intermediacy of a similar species in the phosphorylation of benzyl alcohol and borneol[120] and in another publication, the phosphorylation of 2',3'-O-isopropylidene adenosine.[121] More recently,[56] in studies on the mechanism of the phosphotriester route, 1-methylimidazole has been reported to attack a pyrophosphate in an analogous way to that described by Westheimer.[118]

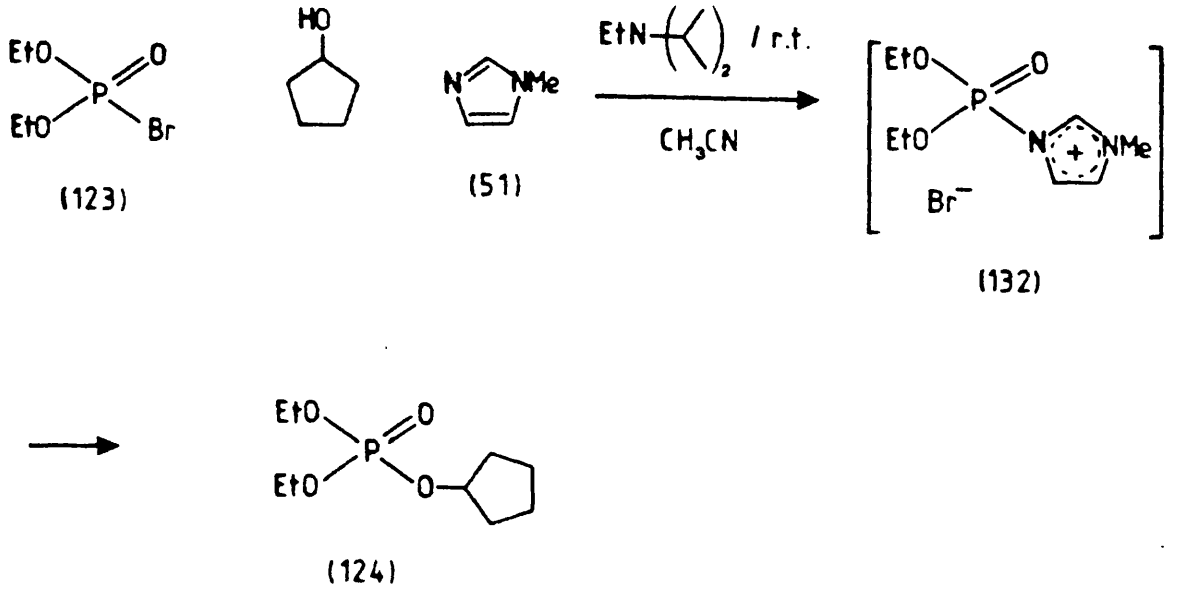
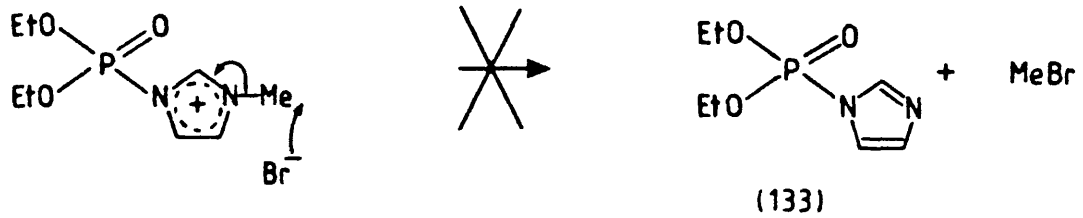


Figure 4.8

Diethyl phosphorobromidate (123) was generated as before by treatment of triethyl phosphite with bromine. The mixture was allowed to warm to room temperature at which point a mixture of cyclopentanol (one molar equivalent) and 1-methylimidazole (51, five molar equivalents) was added. The ^{31}P n.m.r. spectrum recorded within five minutes showed that the signal due to (123) had been replaced by two absorptions at -2.2 p.p.m. and -11.5 p.p.m. present as 75% and 25% of the reaction mixture respectively. The species at -2.2 p.p.m. was isolated in 48% yield and shown to be the required phosphate triester (124).

The reaction had been thought to proceed through the reactive intermediate (132). The identity of the species at -11.5 p.p.m. was, at this point, not known but had been shown not to be a phosphorylating agent as, in a previous experiment, the 1-methylimidazole (51) had been added and the ^{31}P n.m.r. spectrum recorded before the addition of cyclopentanol. The signal due to (123) at -8.7 p.p.m. had been rapidly replaced by the absorption at -11.5 p.p.m. which, subsequently was shown not to react with cyclopentanol over a two hour period.

It was initially proposed that the absorption at -11.5 p.p.m. might be due to demethylation of the intermediate (132) as shown in Figure 4.9. This was subsequently shown not to be the case as when diethyl phosphoro-

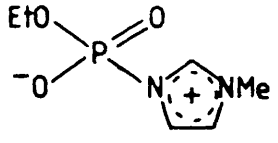


(133)

Figure 4.9

bromidate (123) was treated with imidazole in the presence of base, the ^{31}P n.m.r. spectrum showed the absorption due to (123), -8.7 p.p.m., to have been replaced by an absorption at -6.25 p.p.m., presumably due to (133). In addition, compounds related to (133), where the ethyl groups are replaced by benzyl groups, have been reported to efficiently phosphorylate alcohols.[122]

In retrospect it seems reasonable to suggest that the species having a chemical shift of -11.5 p.p.m. may be (134). This suggestion is based



(134)

on the work of Thuong and Asseline[123] where O,S-dimethyl phosphorochloridate is attacked by 1-methylimidazole (51) displacing Cl^-, which then demethylates the intermediate to give the phosphoimidazolium salt (135), Figure 4.10. The species is reported not to be a phosphorylating agent since in the presence of a 5'-O-protected nucleoside residue in acetonitrile no reaction is observed over a period of three days.

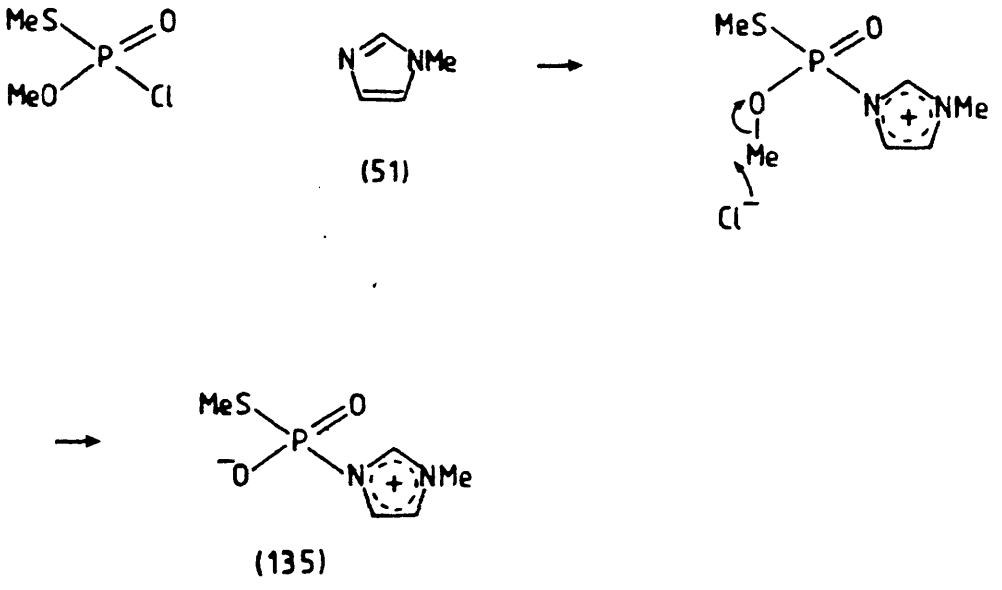


Figure 4.10

However, under optimum conditions in solution, yields of phosphate triesters of up to 70% may be obtained using this procedure, (Figure 3.12, page 79). Therefore, with regard to solid phase synthesis, when an excess of the phosphorylating agent is used then it was expected that side reactions of the type described would be unimportant.

CHAPTER 5

THE PHENYL PROTECTING GROUP AND ACID CATALYSED LIGAND EXCHANGE REACTIONS OF ARYL-CONTAINING PHOSPHITES

The Phenyl Group as the Internucleotide Protecting Group

In addition to the use of the methyl group as the internucleotide protecting group in the proposed Arbusov reaction route it was also intended to investigate the use of the aryl protecting groups, those common in the phosphotriester route, in a similar reaction sequence. Initially, the phenyl group was chosen with a view to progressing on to the o-chlorophenyl group subsequently.

Therefore, the phosphitylating agent required was methyl phenyl phosphorochloridite (136) which was prepared by slow addition of phenol to

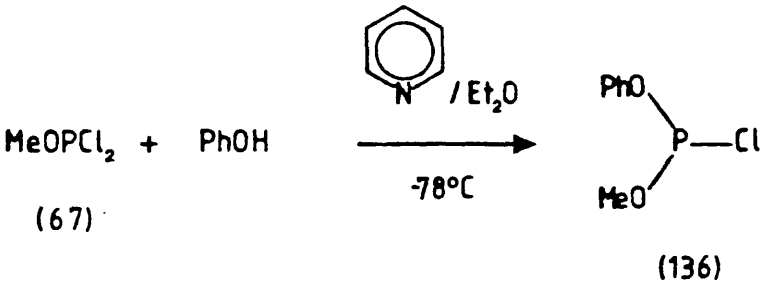


Figure 5.1

a cold ethereal solution of (67) and pyridine, Figure 5.1. (136) was isolated in 83% yield.

The proposed reaction sequence with phenyl as the protecting group is shown in Figure 5.2. It was not expected that attack of bromide ion would occur on the phenyl substituent of the phosphonium halide, the intermediate in the Arbusov reaction, as nucleophilic attack on the aromatic nucleus is extremely slow unless the ring is especially activated.

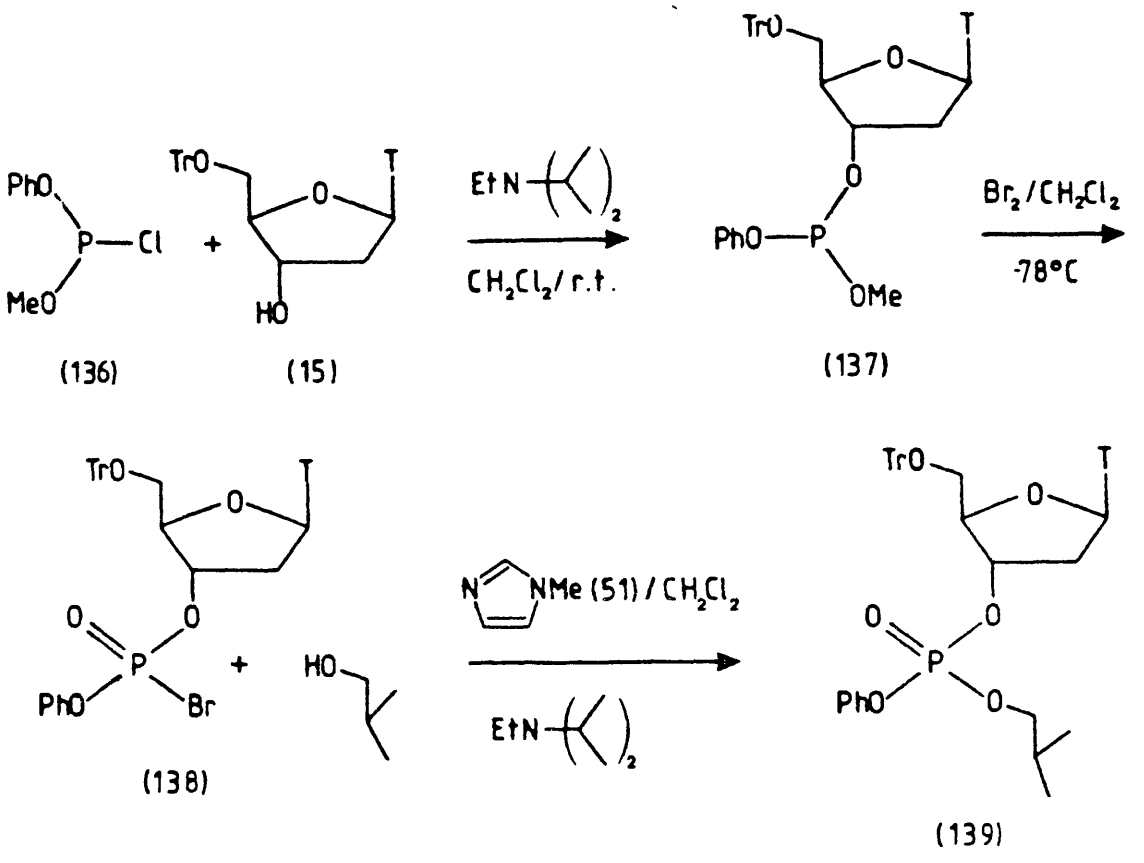


Figure 5.2

Therefore, 5'-O-tritylthymidine (15) was added to one molar equivalent of (136) in the presence of base in methylene chloride solution. The ^{31}P n.m.r. spectrum at the phosphite stage showed a major absorption at +133.9 p.p.m., assumed to be due to the required phosphite (137) although the diastereoisomers were not resolved, and minor absorptions at +127.1 p.p.m. (24% by ^{31}P n.m.r.) and a +5.6 p.p.m. (16% by ^{31}P n.m.r.). The identity of the species at +127.1 p.p.m. is not known but could be due to phosphitylation in the thymine ring; however, no corresponding absorption was observed when the reaction was carried out with dimethyl phosphorochloridite (109). The minor peak at +5.6 p.p.m. is in the region of the spectrum consistant with the product derived from hydrolysis of (136).

One molar equivalent of bromine was added to the solution at -78 ^{o}C. The ^{31}P n.m.r. spectrum recorded within five minutes showed two major

absorptions at -1.2 p.p.m. and -1.6 p.p.m. of comparable intensity. These were assumed to be due to the two diastereoisomers of the phosporobromidate (138). Treatment of (138) with an excess of 1-methylimidazole (51) and one molar equivalent of isobutanol (111) produced a mixture that contained, in the ^{31}P n.m.r. spectrum, a major phosphorus absorption at -7.1 p.p.m. and a minor absorption (broad) at -13.7 p.p.m. (19% by ^{31}P n.m.r.). The species having a chemical shift of -7.1 p.p.m. was isolated, by column chromatography, in 40% yield and was shown to be due to the required aryl dialkyl phosphate (139), it being identical to an authentic sample of (139) prepared by the method of Letsinger,[62] Figure 5.3, starting from phenyl phosphorodichloridite (140).

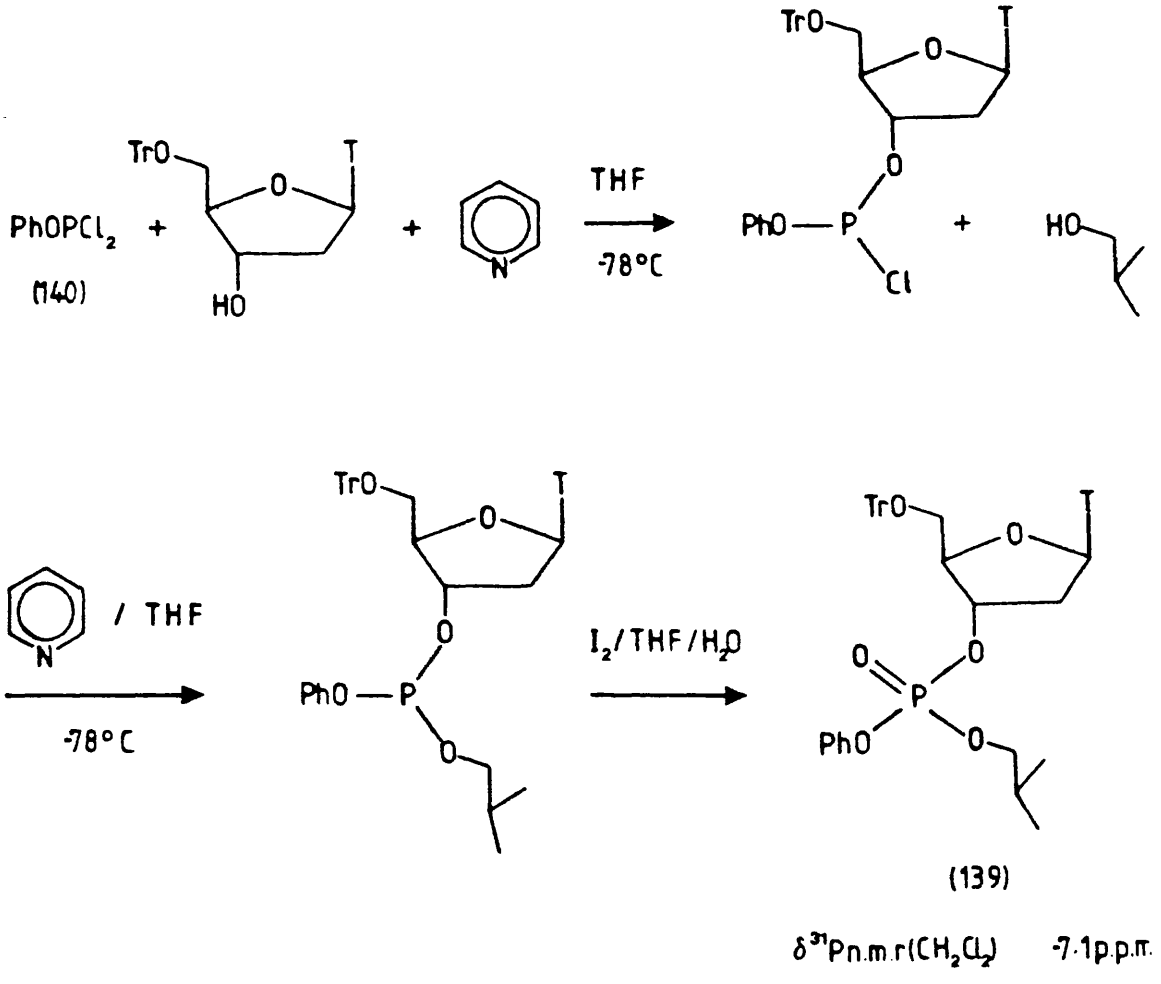
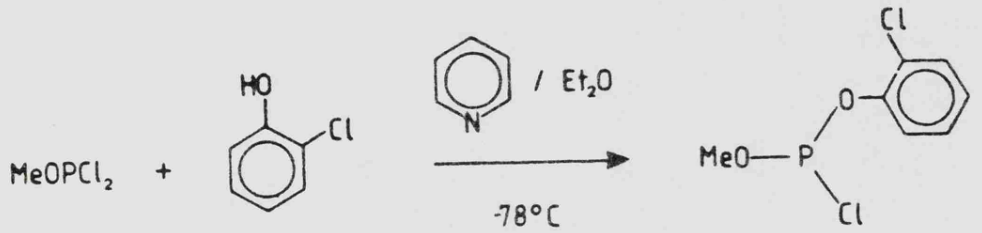


Figure 5.3

The next stage was to prepare the phosphitylating agent containing the o-chlorophenyl substituent, the more common, more easily removed protecting group, this being o-chlorophenyl methyl phosphorochloridite (141). The preparation was attempted in an identical manner to that used to prepare (136).



(67) (141)

Thus, one molar equivalent of o-chlorophenol was added slowly to a solution of methyl phosphorodichloridite and pyridine in ether at -78 °C. The appropriate region of the ^{31}P n.m.r. spectrum is shown in Figure 5.5 (ii). For comparison the corresponding reaction with phenol is shown in Figure 5.5 (i). In the case of o-chlorophenol very poor selectivity for monosubstitution is

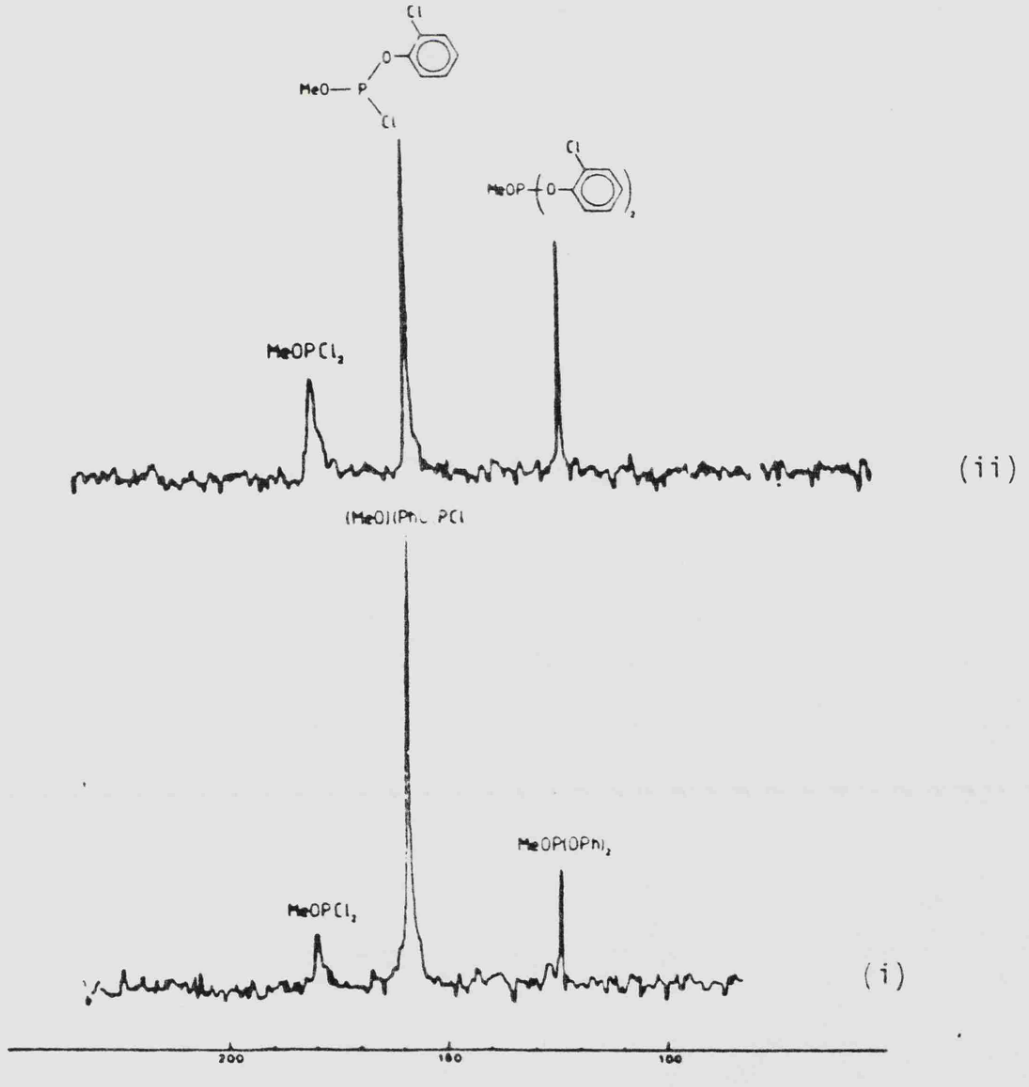
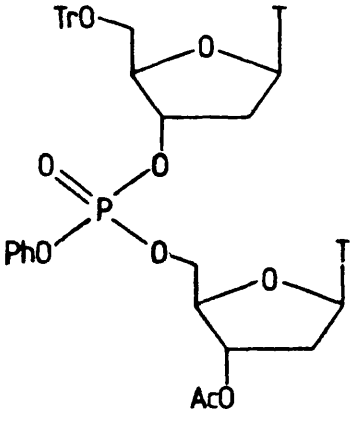


Figure 5.5

observed, much more so than in the case of phenol. Filtration to remove the pyridinium hydrochloride was carried out and the solvent removed under reduced pressure to leave a yellow oil. The ^{31}P n.m.r. spectrum of this oil showed similar characteristics to Figure 5.5 (ii) although the absorption due to di-o-chlorophenyl methyl phosphite (142) at +127 p.p.m. was more intense. Attempted vacuum distillation did not succeed in producing a pure sample of (141), it proving to be thermally unstable; even under very high vacuum. The small amount of distillate obtained was (142). The ^{31}P n.m.r. spectrum of the residue showed a major phosphorus absorption to be at +5.0 p.p.m. with a cluster of minor absorptions between +3.6 p.p.m.and+0.2 p.p.m. Disappointingly, therefore, the phosphorochloridite (141) was found not to be isolable and was not sufficiently pure in solution to proceed on to the phosphite stage, as is evident from Figure 5.5 (ii).

Acid Catalysed Ligand Exchange Reactions

The reaction sequence described in Figure 5.2, with the phenyl substituent present as the protecting group, was repeated with 3'-O-acetyl-thymidine (11a) in place of isobutanol, in the preparation of the fully protected dimer (143). At the phosphite stage of the sequence, in addition



(143)

to the required phosphite (137), the phosphite region of the ^{31}P n.m.r. spectrum was contaminated with three other absorptions at +139.8 p.p.m., +128 p.p.m. and +126.9 p.p.m., present as 12%, 21% and 13% of the reaction mixture (by ^{31}P n.m.r.).

Following the Arbusov reaction and rapid phosphorylation of the 5'-hydroxyl, the ^{31}P n.m.r. of the crude reaction mixture showed two major phosphorus absorptions at -6.9 p.p.m. and -7.2 p.p.m. Following isolation (24%) they were shown to be due to the two diastereoisomers of the required phosphate (143). The spectrum also contained several peaks, as minor impurities, between -11.7 p.p.m. and -14 p.p.m., the region of the spectrum usually associated with pyrophosphate species.

The low yield of the required phosphate is not surprising considering that at the phosphite stage, (137) was present as only 47% (by ^{31}P n.m.r.) of the mixture.

It was not immediately obvious how the extra phosphorus (III) species arose in the ^{31}P n.m.r. spectrum during the reaction sequence. They could arise from an acid catalysed ligand exchange process resulting in structural reorganisating to give a mixture of phosphites. Moedritzer[124] has observed similar behaviour when trimethyl and triethyl phosphite are mixed together with HCl as catalyst. However, 10-15 hours were required to bring about equilibrium in the exchange of alkyl groups.

In the reaction sequence described in Figure 5.2, however, a phenyl group is present as one of the substituents. Phosphites containing phenyl substituents are reputed to undergo an exchange process more rapidly than alkyl substituents as Rydon[125] reported the ease of reaction of glycols with

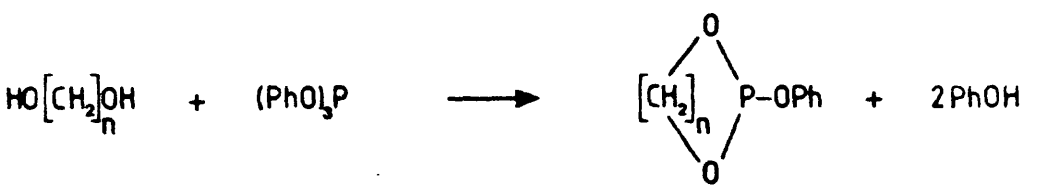


Figure 5.6

triphenyl phosphite, Figure 5.6, a reaction that proceeds more rapidly in the presence of an acid catalyst. This, therefore, suggested an explanation for the experimental observation. As the major species present at the phosphite stage of the sequence of reactions in Figure 5.2 was that due to the required phosphite, under these conditions the exchange process is not particularly rapid. However, if this process could be accelerated it could be used as a new phosphite forming reaction. In solution, once the phenyl group had been exchanged subsequent scrambling of alkyl ligands can occur to give a statistical mixture of all possible phosphites as has been observed by Engels[82] Figure 2.6, page 47. However, this was not regarded as a problem in solid phase synthesis.

In principle, therefore, two adjustments could be made that might result in overall acceleration of the exchange reaction. Firstly, variation of the acid catalyst and secondly variation of the aryl grouping.

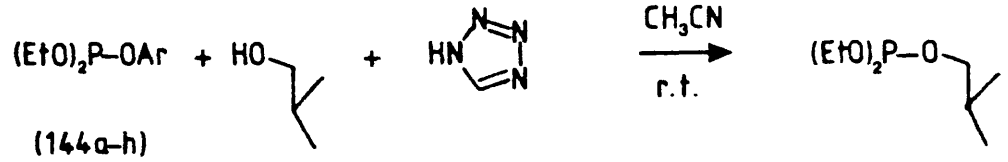
The catalyst used must not be too acidic since acid labile protecting groups are employed at the 5'-hydroxyl position. In his observation of this exchange phenomenom Engels[82] reported tetrazole (72) to be an efficient catalyst and since it is known not to be acidic enough to result in detritylation, as it is used in the phosphoramidite route, tetrazole seemed to be a logical choice.

It was imagined that if electron-withdrawing substituents were incorporated into the aryl ring then the P-OAr bond would be weakened, thus rendering the substituted aryl group a better leaving group. Phenols containing progressively more electron-withdrawing substituents became progressively more acidic. Therefore, the lower the pK_a of the phenol used, the better its leaving ability should be.

To test this idea exchange reactions were carried out on a series of simple aryl diethyl phosphites (144 a-h) with isobutanol (111), as a model

for the 3'-O-protected nucleoside derivative, using tetrazole (72) as acid catalyst. The phosphites used, and the relative rates of exchange are compiled in Figure 5.7.

The reactions were monitored by ^{31}P n.m.r., and following the disappearance of the starting material, the product, diethyl isobutyl phosphite (145), was oxidised with sulphur to the corresponding thiophosphate



(144a–h)

(145)

T = Total time for complete exchange

Ar		pK_a of free alcohol	T
p-MeO Ph	(144a)	10·21	26 hours
Ph	(144b)	9·9	
p-Cl Ph	(144c)	9·2	
o-Cl Ph	(144d)	8·5	Increasing Rate of Exchange
3,5-DiCl Ph	(144e)	7·9	
2,4-DiCl Ph	(144f)	7·5	
2,6-DiCl Ph	(144g)	6·7	
p-NO_2 Ph	(144h)	7·15	25 minutes

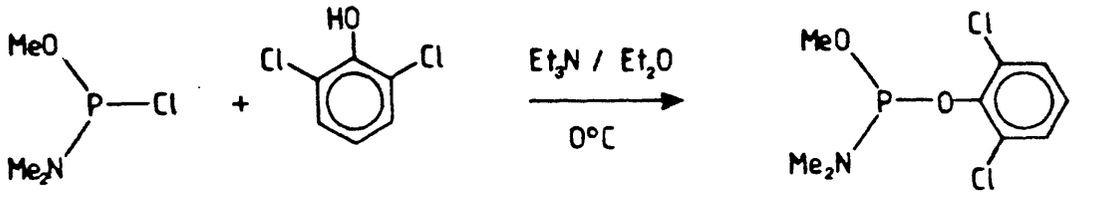
Figure 5.7

and isolated as such for identification.

The trend of the experimental observations seems consistent with the general ideas presented above. However, p-nitrophenyl exchanges more rapidly than does 2,6-dichlorophenyl even though it is not as acidic. As is suggested on page 48, the exchange mechanism may be bimolecular. This being the case, then the slower rate of exchange of the 2,6 -dichlorophenyl group may be due to steric effects.

However, with a view to using this reaction as a phosphite forming reaction with potential for oligonucleotide synthesis, a phosphitylating agent containing the exchangeable group was required. It had already been determined that the isolation of phosphorochloridites containing chlorine-substituted aryl groups was not possible. Therefore, the phosphite precursor needed to be phosphoramidite.

Ideally, therefore, the best exchangeable group to use would be p-nitrophenyl. However, the heating of a phosphorus (III) compound in the presence of an aromatic nitro compound produces an aryl nitrene.[126] This, therefore, meant that a phosphoramidite containing a p-nitrophenyl group would not be distillable and could not be obtained pure. The next best exchange grouping is the 2,6-dichlorophenyl group. Therefore, 2,6-dichlorophenyl methyl N,N-dimethylphosphoramidite (146) was prepared from chloro-N,N-



(70)

(146)

δ^{31}P n.m.r(Et_2O) +150p.p.m

Figure 5.8

dimethylaminomethoxyphosphine (70) and 2,6-dichlorophenol, as in Figure 5.8, and obtained in a crude yield of 71% A small amount was distilled prior to use in the phosphite forming reaction. Figure 5.9 shows the reaction scheme through to the trialkyl phosphate (112a). 5'-O-dimethoxytrityl-thymidine (10a) was added to one molar equivalent of (146) and tetrazole (72) in acetonitrile solution. The ^{31}P n.m.r. spectrum showed four major phosphorus absorptions at +137.1 and +136.1 p.p.m. assumed to be due to the two

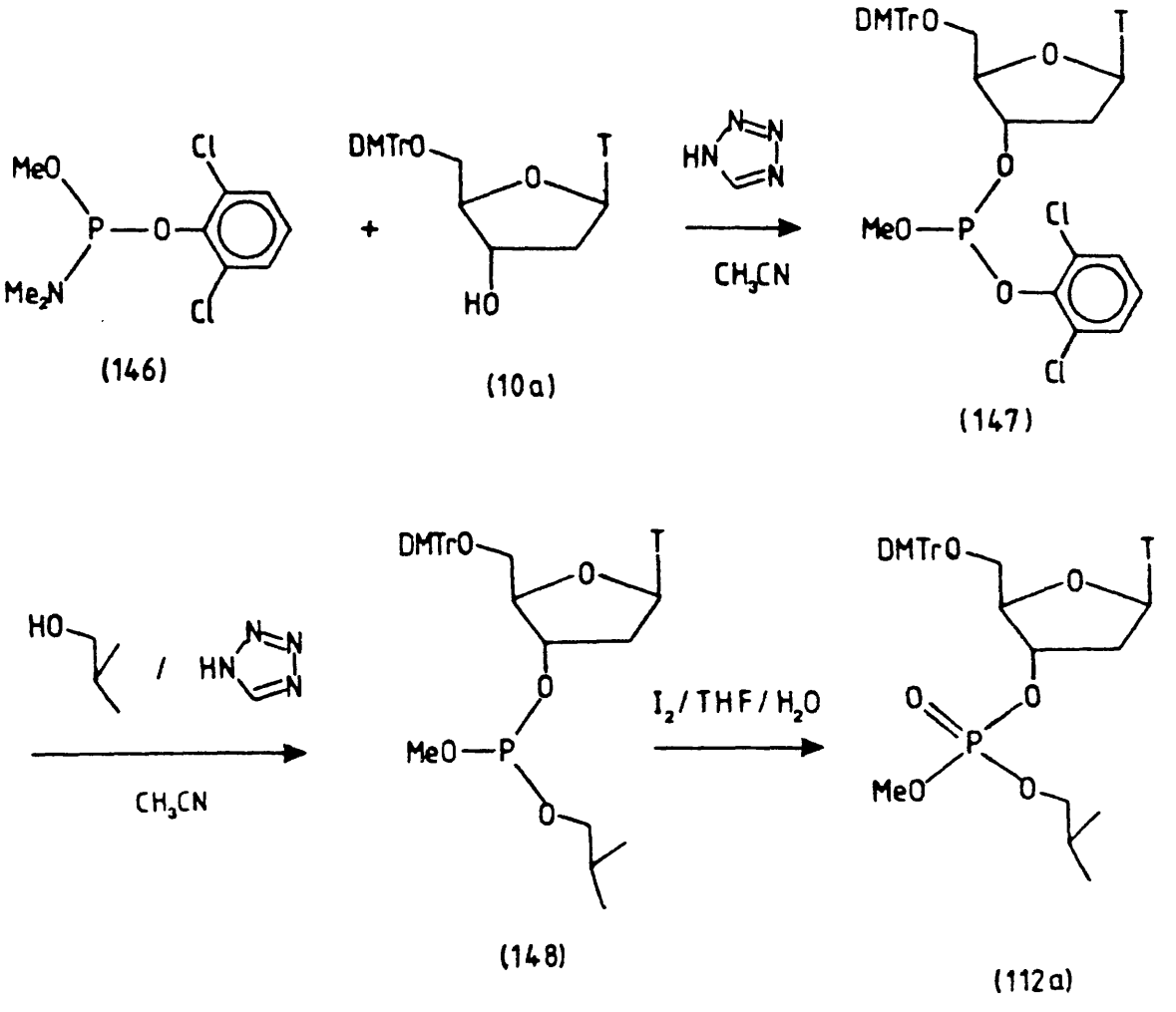


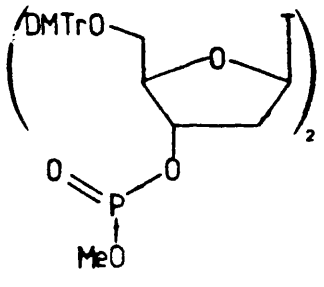
Figure 5.9

diastereoisomers of the phosphite (147) and at +8.9 p.p.m. and +4.2 p.p.m., species that may be hydrolysis products. Isobutanol and four molar equivalents of tetrazole were added and the exchange process monitored by ^{31}P n.m.r. It took approximately 90 minutes for the peaks at +137.1 p.p.m. and +136.1 p.p.m. to be completely replaced by a broad absorption at +139.2 p.p.m., presumably due to the phosphite (148), although the diastereoisomers were not resolved. In addition, the absorptions present at +9.0 p.p.m. and +4.4 p.p.m. were still present as 31% and 35% of the reaction mixture (by ^{31}P n.m.r.) respectively.

The mixture was treated with the standard aqueous iodine oxidising solution. Following this, the ^{31}P n.m.r. spectrum showed no absorptions in

the phosphite region of the spectrum but a major phosphorus absorption at -1.2 p.p.m. with several minor peaks present at +9.6, +6.1, +0.2, -0.2, -2.4 and -10.5 p.p.m. Following work-up and removal of solvent the ^{31}P n.m.r. contained three major phosphorus absorptions at 0.0 p.p.m., -1.0 p.p.m. and -2.0 p.p.m. present as 16%, 53% and 32% (by ^{31}P n.m.r.) of the mixture respectively. After preparative thin layer chromatography on silica gel the species at -1.0 p.p.m., the absorption of which was now shifted to -0.4 p.p.m. as the solvent had changed, was shown to be the required phosphate (112a), it being identical by ^{31}P n.m.r., ^{1}H n.m.r., and tlc to the sample of (112a) prepared by the Arbusov reaction route. The yield was only 20%.

The species at -2.0 p.p.m. was shown by ^{1}H n.m.r. to be due to the (3'-3') symmetrical dinucleoside phosphate (149).



(149)

From this series of reactions, although a small amount of the required product had been obtained, the indications were that this method is not suitable to attempting the synthesis of oligonucleotides, for several reasons.

(i) The starting phosphoramidite is very sensitive to hydrolysis. Therefore, in the phosphitylation step, there remains a substantial amount of the 5'-protected nucleoside derivative not phosphitylated.

(ii) For a practical synthesis, the phosphite would need to be prepared and stored until required. However, as the phosphite is prepared from a phosphoramidite, tetrazole is present to catalyse the phosphitylation. Subsequent ligand exchange would be expected due to the presence of tetrazole during prolonged storage,

(iii) The exchange reaction itself, in solution, is not particularly rapid. Therefore, it would not be expected to proceed rapidly in a heterogeneous reaction on a solid support.

Therefore, of the two methodologies investigated, the Arbusov reaction route seemed to have much more potential with regard to solid phase synthesis than did the exchange reaction route.

CHAPTER 6

APPLICATION OF THE PROPOSED ARBUSOV REACTION ROUTE TO SOLID PHASE SYNTHESIS

Application of the Proposed Arbusov Reaction Route to Solid Phase Synthesis

The rapid, stepwise, chemical synthesis of oligonucleotides on an inorganic polymer support has been successfully achieved using both the phosphotriester route and the phosphoramidite route and has culminated in the commercial availability of automated DNA synthesisers using both methodologies.

Having developed a relatively efficient coupling reaction in solution involving the Arbusov reaction it was necessary, for it to be a viable alternative, to apply the procedure to solid phase synthesis.

The Support

The solid phase method involves repeated cycles of addition to the solid support of a solvent or reagent, the excess of which is removed by washing. In practice, the most efficient way to wash a support is to pack it into a column and flow solvents and reagents through it. The experience of Gait and Sproat[54] is that highly swollen gel resins are not ideal for such a system and fine mesh materials can cause clogging of frits and therefore generate back-pressures. The recommended supports for use in the system described are long chain alkylamine controlled-pore glass (LCAA/CPG), polydimethylacrylamide-Kieselguhr and Whatmann 3MM chromatography paper.

LCAA/CPG is ideal in that it is chemically inert, non-swellable and relatively non-polar and it has been used by Gait and Sproat[54] in the preparation of a purine-rich 37-residue oligonucleotide in good yield using the phosphotriester approach and a 51-residue oligonucleotide has been prepared by Adams et al[127] using the phosphoramidite approach. Fully functionalised supports are commercially available (e.g. from Crauchem, Scotland) and a batch of LCAA/CPG functionalised with thymidine as the first nucleo-

side residue, attached to the LCAA spacer via a succinate linkage, Figure 6.1, was made available by Celltech Ltd. (Slough, Berkshire).

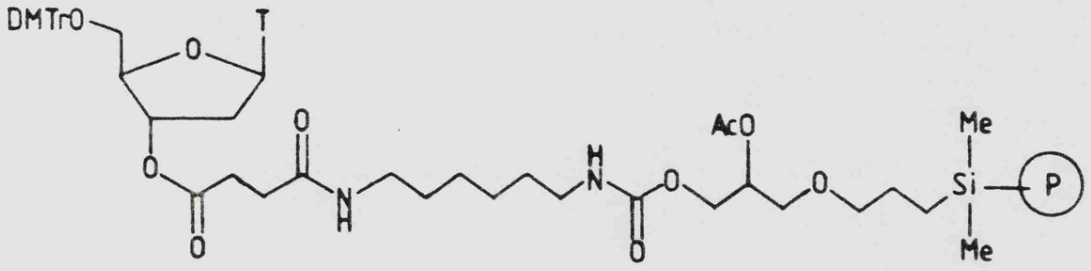


Figure 6.1

The Manual Synthesis Arrangement

An omnifit column (Omnifit Ltd., Cambridge, U.K.), 38 x 7 mm, i.d., was used in which to pack the LCAA/CPG support. A schematic diagram of the

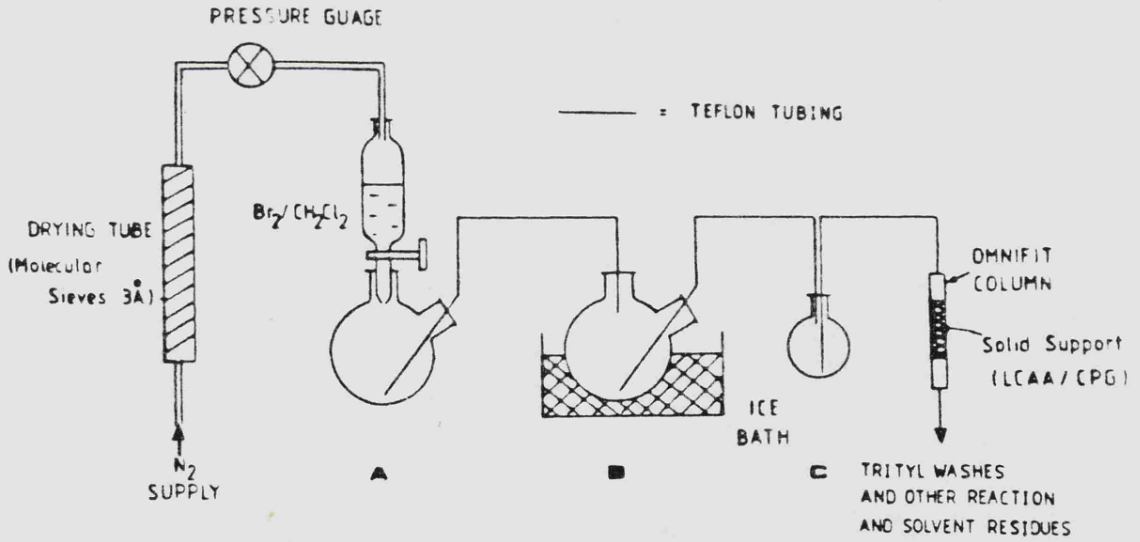


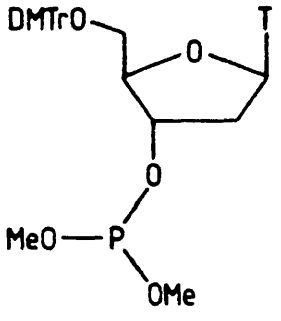
Figure 6.2

manual synthesis apparatus is shown in Figure 6.2.

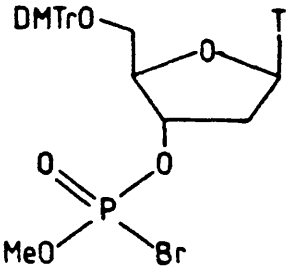
A pressure of dry nitrogen (a drying tube was inserted as a precaution against moisture getting into the system) was used to force reagents through the apparatus. Flexible teflon tubing, cut to the required length so as to minimize the amount of dead volume, was used to transport

solvents and reagents throughout the apparatus. The teflon tubing was pushed through rubber septum caps to allow entry into each flask.

Flask A contained a solution of bromine in, for the most cases, methylene chloride which, under a pressure of nitrogen flowed into flask B containing the dimethyl nucleoside phosphite (107a), thus generating the phosphorobromidate (110a). This was then transported to flask C, containing



(107a)



(110a)

1-methylimidazole (51) and diisopropylethylamine, to generate the active phosphorylating agent just prior to addition to the column. Washing solvents and other reagents were injected into flask C by syringe through a rubber septum cap as required.

General Procedure for Chain Assembly

The synthesis of oligonucleotides using the chemistry described was attempted in a cyclical way. Basically, three chemical reactions were involved per cycle, Figure 6.3. In the first the Arbusov reaction on the phosphite (107a) gives the phosphorobromidate (110a). The second is the removal of the 5'-protecting group (DMTr) by a suitable protic acid, namely 10% trichloroacetic acid in methylene chloride [conversion of (150) into (151)]. After appropriate solvent washes of the support to remove residual acid, the phosphorobromidate (110a) is mixed with 1-methylimidazole (51)

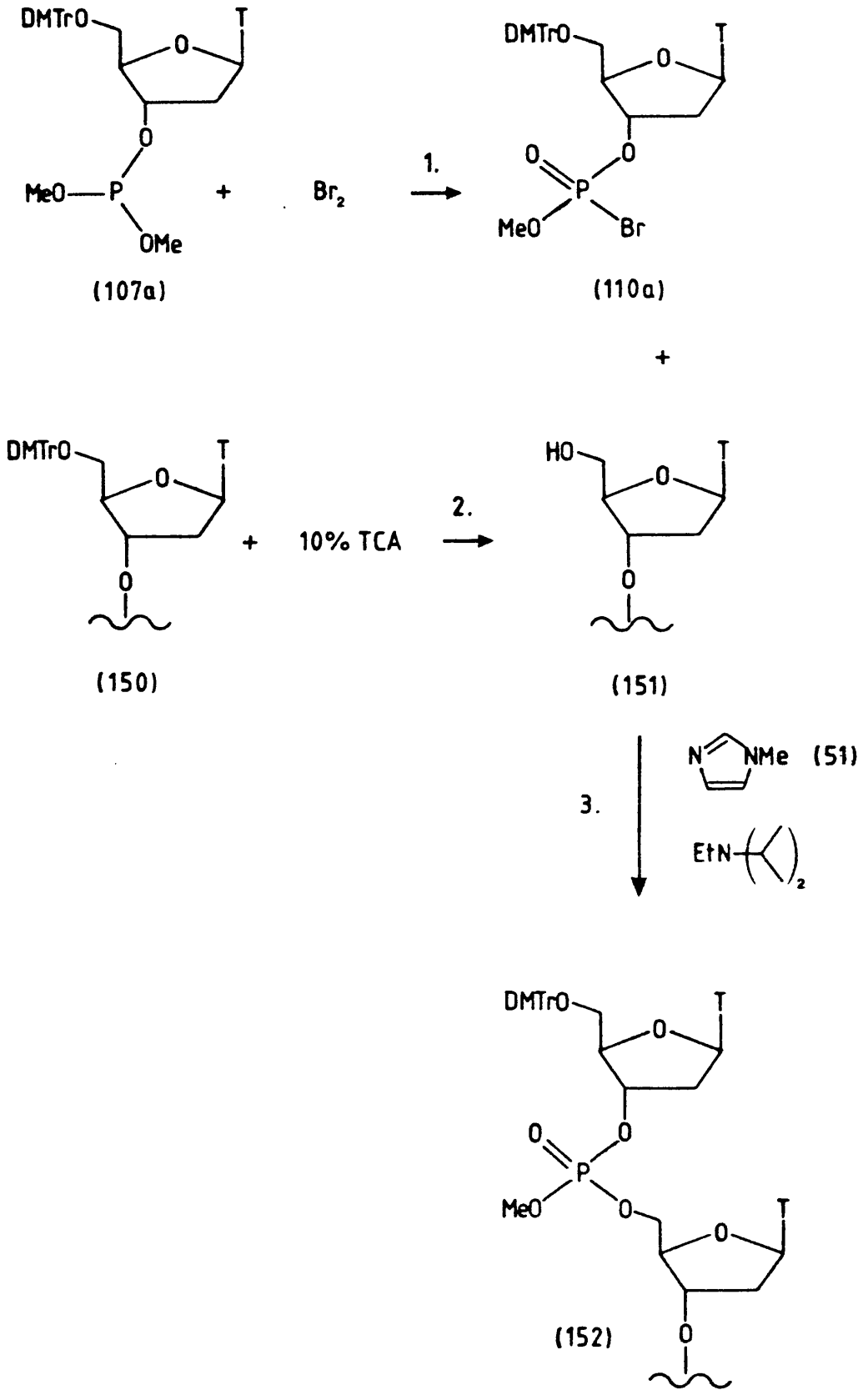
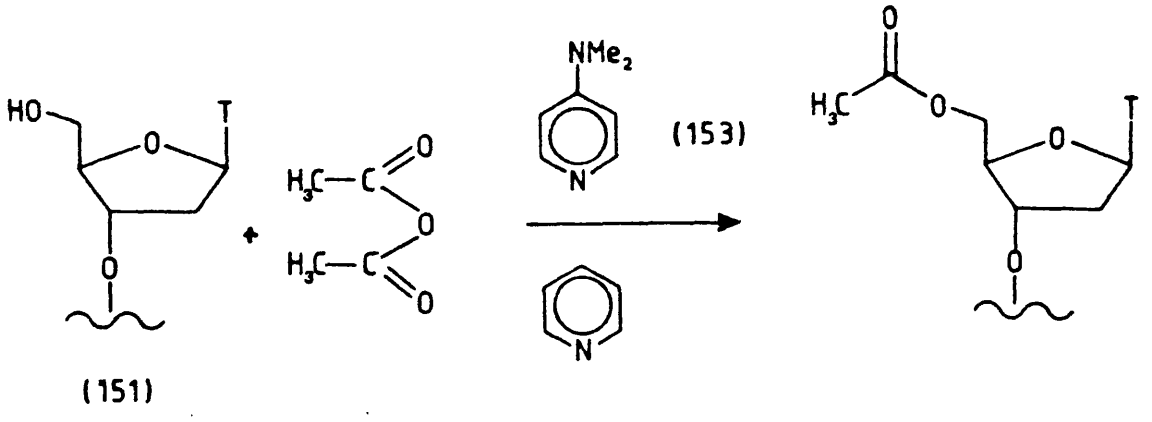

DMTrO
T
O
O
MeO—P
OMe
(107a)
+
Br2
1.
DMTrO
T
O
O
O
P
MeO
Br
(110a)
+
DMTrO
T
O
O
(150)
+
10% TCA
2.
HO
T
O
O
(151)
3.
N
NMe
(51)
EtN
2
DMTrO
T
O
O
O
P
MeO
O
T
O
O
(152)

Figure 6.3

in the presence of base and, in the third reaction, passed over the polymer-bound nucleoside residue with a free 5'-hydroxyl (151). A 'capping' step is available, which acylates any 5'-hydroxyl groups that have not been



(151)

Figure 6.4

phosphorylated, Figure 6.4, thus rendering them unreactive in subsequent phosphorylations. This prevents the gradual construction of shorter, undesired oligomers in addition to the required sequence, a condition that would make the ultimate purification more awkward. However, as only thymidine containing phosphorylating agents were to be used, this option was not initially employed. Extension of the chain is effected by 5'-deprotection, washings, and subsequent phosphorylation. A typical cyclical sequence is tabulated in Figure 6.5.

1. Pyridine } Wash
2. CH_2Cl_2 }
3. 10% TCA 1 minute
4. CH_2Cl_2 wash
5. Phosphorylation, (109a) + (51) + DIPEA
6. CH_2Cl_2 wash
7. 10% TCA

etc

Figure 6.5

Although the phosphorylation reaction is rapid in solution, there was no guarantee it would be so in the solid phase synthesis. The reaction time for step 5, therefore, needed to be determined by experiment. Initial reaction times employed for step 5 were of the order of 30 minutes.

The dimethyl nucleoside phosphite (107a) was prepared as described, Figure 3.12, page 79, the day prior to the solid phase experiment, made up to a known concentration and stored in a storage vial overnight in the deep freeze. The conversion of (107a) into the phosphorobromidate (110a) was checked by ^{31}P n.m.r. spectroscopy before addition to the column and in most cases was found to be the major absorption in the ^{31}P n.m.r. spectrum at +4.6 p.p.m, although in some cases a significant amount of material with an absorption of -12.5 p.p.m., assumed to be due to pyrophosphate species, was observed.

Deprotection and Analysis

After the appropriate number of cycles of deprotection and phosphorylation, the crude product is released into solution following three further operations, Figure 6.6. Firstly, the internucleotide methyl esters are removed by treatment with thiophenol (154) and triethylamine in dioxan solution.[30] Secondly the oligomer is released from the support by treatment with concentrated ammonia at 50 ^{o}C in a sealed vial. After evaporation of the solution the residue is treated with 80% acetic acid to remove the 5'-terminal protecting group.

The use of a high performance liquid chromatography (hplc) has resulted in oligonucleotides separation with a high degree of efficiency. Oligonucleotides are polyanions with lipophilic bases. Therefore, anion-exchange and reversed-phase chromatography are effective for their purification. Reversed-phase chromatography can be compared to traditional adsorption-chromatography which is carried out on a polar stationary phase

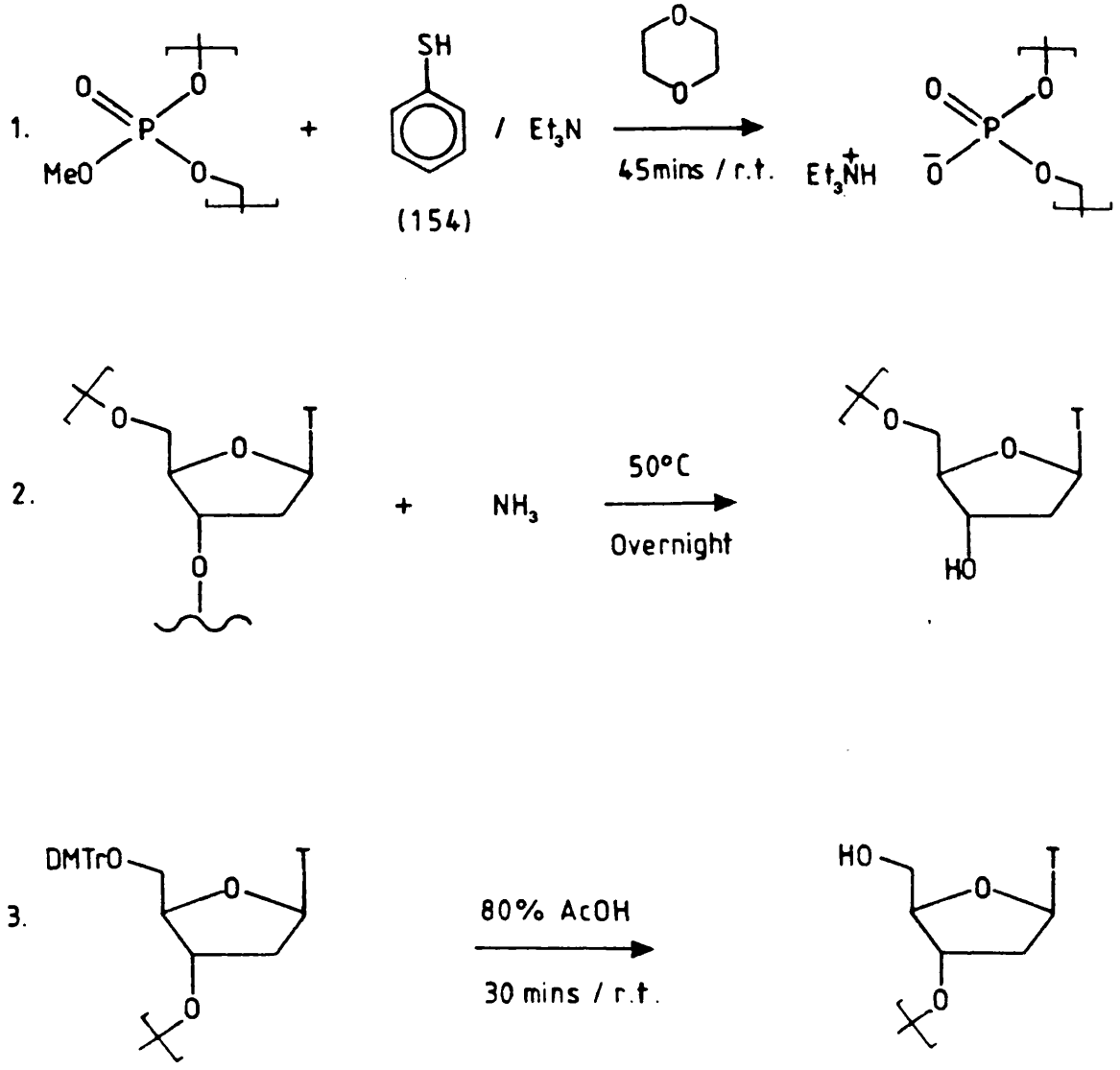


Figure 6.6

such as silica gel and uses a non-polar mobile phase. In reversed-phase chromatography the stationary phase is non-polar and the mobile phase polar and it is the non-polar interactions which determine the migration velocity along the stationary phase. Therefore, polar solutes are eluted earlier from the column than non-polar solutes.

In anion-exchange chromatography it is the extent of the adsorption-desorption of the anionic solute on the cationic stationary phase that determines the migration velocity. The adsorption and desorption processes depend upon the nature and concentration of the eluting buffer. The elution of polyanions, such as oligonucleotides occurs according to the number of

anionic charges, generally reflecting the length of the polymer.

Initially, anion-exchange hplc was carried out on a Partisil 10 SAX column (25 cm x 0.7 cm) using the conditions of Eaton;[128] a column temperature of 60 ^{o}C using a gradient elution of water: acetonitrile: 3M triethylammonium formate, pH 5.0, from 57:25:18 to 39:25:36 over 60 munutes. Using these conditions, no separation was effected, all u.v. absorbing material (at 260 nm) eluting with a retention time of 1-2 minutes. Therefore, the conditions of Gait were employed[129] where chromatography is carried out with a column temperature of 25 ^{o}C using buffers of 1mM and 300 mM KH_2PO_4, pH 6.3, in formamide:water (6:4), the gradient running from 100% 1mM, 0% 300 mM to 0% 1 mM, 100% 300 mM over 60 minutes. The formamide prevents aggregation phenomena which can be a nuisance with G-rich sequences. However, the presence of formamide resulted in sloping base lines and as no G was present, formamide was subsequently not used.

Initial solid phase experiments were carried out in an attempt to prepare a hexamer containing thymidine only, (TpTpTpTpTpT). This, therefore involved, five deprotections and five phosphorylations in the synthesis cycle prior to the final deprotections. During the experiment, the information given by the trityl cation assay was very indeterminate suggesting incomplete detritylation at one step and excessive detritylation at the subsequent step.

H.P.L.C. analysis of the fully deprotected material showed three peaks with retention times of 1.7 minutes (71%), 2.2 minutes (21%) and 8.9 minutes (8%). Authentic T, unphosphorylated, eluted with a retention time of 1.7 minutes, (TpT) with a retention time of 2.6 minutes and (TpTpT) at 9.5 minutes. Therefore, the required oligomer would be expected to elute with a retention time far greater than those observed in the crude reaction mixture.

It was evident that the majority of the mixture contained thymidine that had not been phosphorylated. This meant that the rapid phosphorylation

observed in the solution was not occurring in the solid phase experiments. The only other alternative was that the required product had been formed but had been degraded in the final deprotection procedures. As these were common, standard, deprotection reactions, this alternative was not thought to be probable.

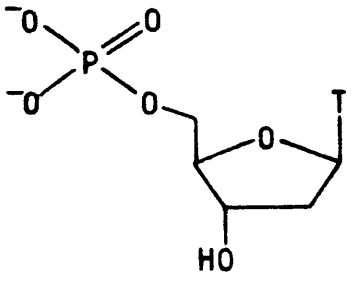
In a subsequent experiment, aimed at the synthesis of the trimer (TpTpT), thus two phosphorylations, the concentration of the phosphorobromidate (110a) was increased, as was the reaction time of the phosphorylation reaction, from 30 minutes to 50 minutes. In addition, a polar solvent (acetonitrile) wash was incorporated into the cycle in case the methylene chloride washing step was not sufficient to wash clear of the support any reaction residues. Prior to the first phosphorylation, trityl cation analysis indicated a quantitative deprotection of the first residue. Following the first phosphorylation, the second deprotection assay indicated a 75% coupling efficiency. Following further phosphorylation and final deprotection, the hplc chromatogram of the crude material contained a major peak with a retention time of 1.9 minutes, with minor peaks eluting after 5.6 minutes and 7.9 minutes. Authentic thymidine, r.t. 1.9 minutes, (TpT), r.t. 3.9 minutes and (TpTpT), r.t. 7.0 minutes were used as standards.

Thymidine appeared to be the major component of the mixture indicating very poor phosphorylation. This was very odd considering the trityl cation assay. Experiments thereafter were conducted with the intention of making sure that the first coupling was effective.

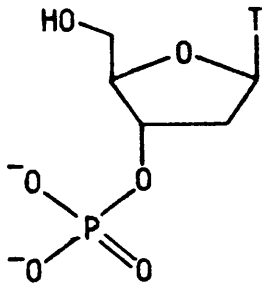
Repeated solid phase experiments were carried out with various modifications being employed. In different experiments phosphorylations were carried out in pyridine and acetonitrile as solvent with varying degrees of concentration of the phosphorobromidate and various reaction times. However, no dramatic differences in the hplc traces were observed. DMF was

introduced, in addition to the acetonitrile polar wash step, but had no notable effect. Various excesses of the phosphorobromidate were used from 30 molar equivalents per phosphorylation to 188 molar equivalents, again with no beneficial effect.

If the required oligomers were being formed but being degraded in the terminal deprotection steps, then the hplc, in addition to just thymidine, ought to contain peaks due to thymidine 5'-monophosphate (155) and thymidine



(155)



(156)

3'-monophosphate (156). Samples of both were analysed under the hplc conditions used to examine the reaction mixtures and were found not to coincide with peaks in the reaction mixture chromatograms.

The best coupling obtained in the solid phase experiments was in an attempted synthesis of (TpT) which, after analysis by hplc, was found to constitute only 34% of the product mixture, calculated by peak areas.

The degree of phosphorylation observed was obviously not acceptable. Despite variation of solvents, concentrations, reaction times and washing steps it had become quite apparent that the ease of phosphorylation of alcohols in solution using the phosphorobromidates (110a-d) was not easily transferable to solid phase synthesis.

Conclusions and Suggestions for Further Research

The Arbusov reaction on dimethyl nucleoside phosphites (107a-d) has been shown to proceed rapidly with loss of methyl bromide to give the phosphorobromidates (110a-d). In solution, in the presence of 1-methylimidazole (51) and an alcohol, rapid phosphorylation of that alcohol is effected.

The procedure developed in solution has been shown not to operate successfully in solid phase synthesis. The longer reaction times that seem to be required in solid phase synthesis compared to the solution counterparts may be of sufficient duration that a fate other than phosphorylation of the 5'-hydroxyl groups may have befallen the activated phosphorobromidate. A possible explanation is suggested from the work of Thuong and Asseline,[123] Figure 4.10, page 96 in the formation of the phosphoimidazolium salt (135). The corresponding transformation in our experiments is shown in Figure 6.7.

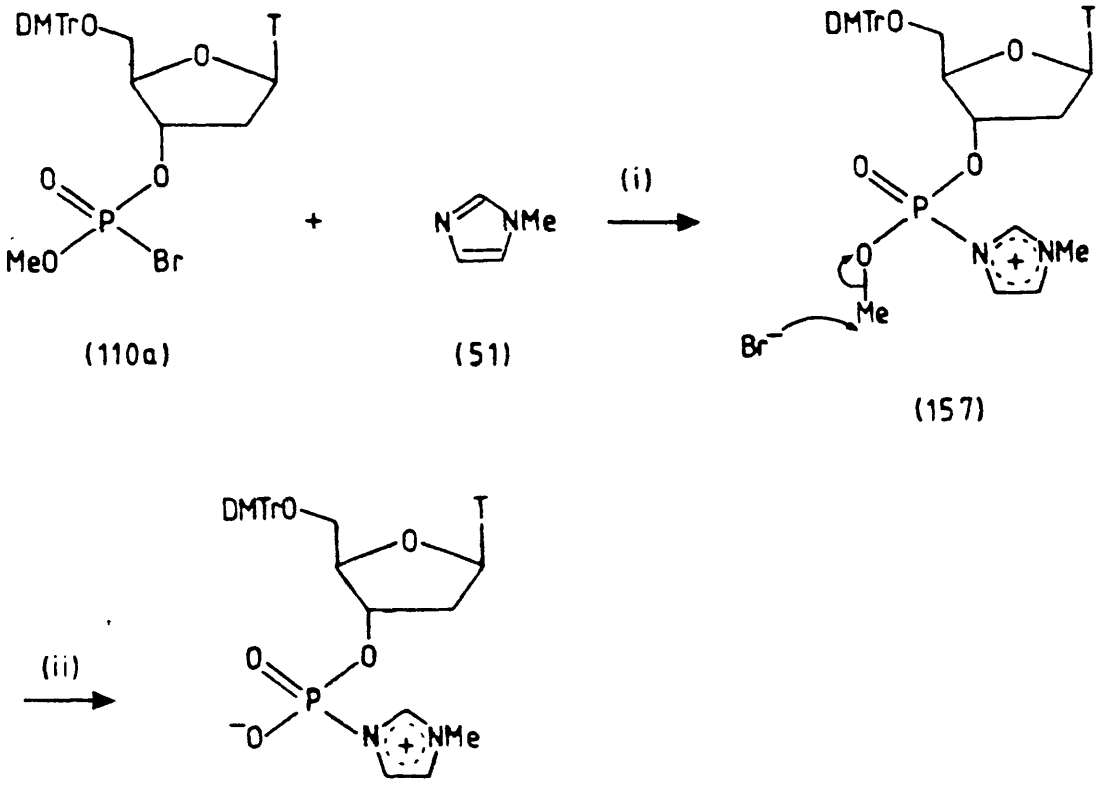
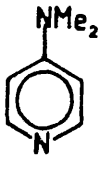


Figure 6.7

Since the activated phosphorobromidate (157) is assumed to be the intermediate in the solution phosphorylation reactions then step (i) is expected to be rapid. The phosphorylation of the alcohol in solution is of such rapidity that the proposed step (ii) is not in serious competition. In the solid phase experiment the phosphorylation seems to be slower to the extent that step (ii) becomes a serious competitor and results in the formation of substantial amounts of (158), the analogue of which (135), page 96 is reported not to be a phosphorylating agent.[123] This would account for the poor phosphorylating ability on the solid support.

It is not easy to see how to circumvent this problem. Alteration of the activating agent may have some effect. For example, N,N-dimethylaminopyridine, DMAP (153), has been reported to act in a similar way to 1-methyl-



DMAP (153)

imidazole (51).[55] However, whatever the activating agent used, bromide ion is displaced and therefore available to attack the methyl group.

The use of less nucleophilic halide, Cl^-, thus hoping to make step (ii) slower was not expected to assist matters since the publication that reports the analogue of (158) does not begin from a phosphoryl bromide but a phosphoryl chloride.

Although phosphite chemistry is used in the proposed approach, the actual coupling reaction is a phosphorylation, not a phosphitylation. In this respect, the approach proposed bears closest resemblance to the phosphotriester route, but with a methyl protecting group. The phosphotriester route has not been reported with a methyl internucleotide protecting group[130] although Gait has reported that methyl-substituted phosphorylating agents are poorly reactive.[131] In the same report, chlorophenyl-containing

phosphitylating agents are stated as being impossible to prepare suggesting the protecting groups between the different chemistries are not interchangeable although a recent report[132] has succeeded in utilising a phosphoramidite containing an o-chlorophenyl substituent in solid phase synthesis.

To use successfully the proposed Arbusov reaction approach on solid supports would, it seems, require the presence of an alternative protecting group to methyl, e.g. the o-chlorophenyl group. Although phosphitylating agents containing this substituent are said to be impossible to prepare, a possible way of carrying out the required procedure is shown in Figure 6.8. The proposed procedure, uses Caruthers' bisalkylaminophosphine (76)[68]. This was prepared from methyl phosphorodichloridite (67) and diisopropylamine and isolated in 52% yield. Therefore, one molar equivalent of the 5'-protected nucleoside derivative (10a) and one molar equivalent of the amine salt (77) was added to (76). The ^{31}P n.m.r. spectrum of the resulting mixture showed three major absorptions at + 149.4 p.p.m., + 149.1 p.p.m. [the diastereoisomers of the phosphoramidite (74a)] and at + 14.5 p.p.m., probably due to hydrolysis. Addition of one molar equivalent of o-chlorophenol and a further molar equivalent of (77) produced a reaction mixture that contained, in the ^{31}P n.m.r. spectrum, four major absorptions at +135.5 p.p.m., +134.5 p.p.m., +134.1 p.p.m. and +14.9 p.p.m. and a minor absorption at +8.7 p.p.m. Bromine addition at 0 ^{o}C succeeded in replacing the signals in the phosphite region of the spectrum with a new absorption at -6.7 p.p.m., speculated to be due to the o-chlorophenyl containing phosphorobromidate (160), although this absorption was a minor component of the mixture, the major absorptions being at +15.1 p.p.m. and +8.7 p.p.m. In the solid phase experiment, this mixture, together with the appropriate amounts of 1-methylimidazole (51) and diisopropylethylamine, was passed over the deprotected nucleoside unit attached to the LCAA/CPG support (151).

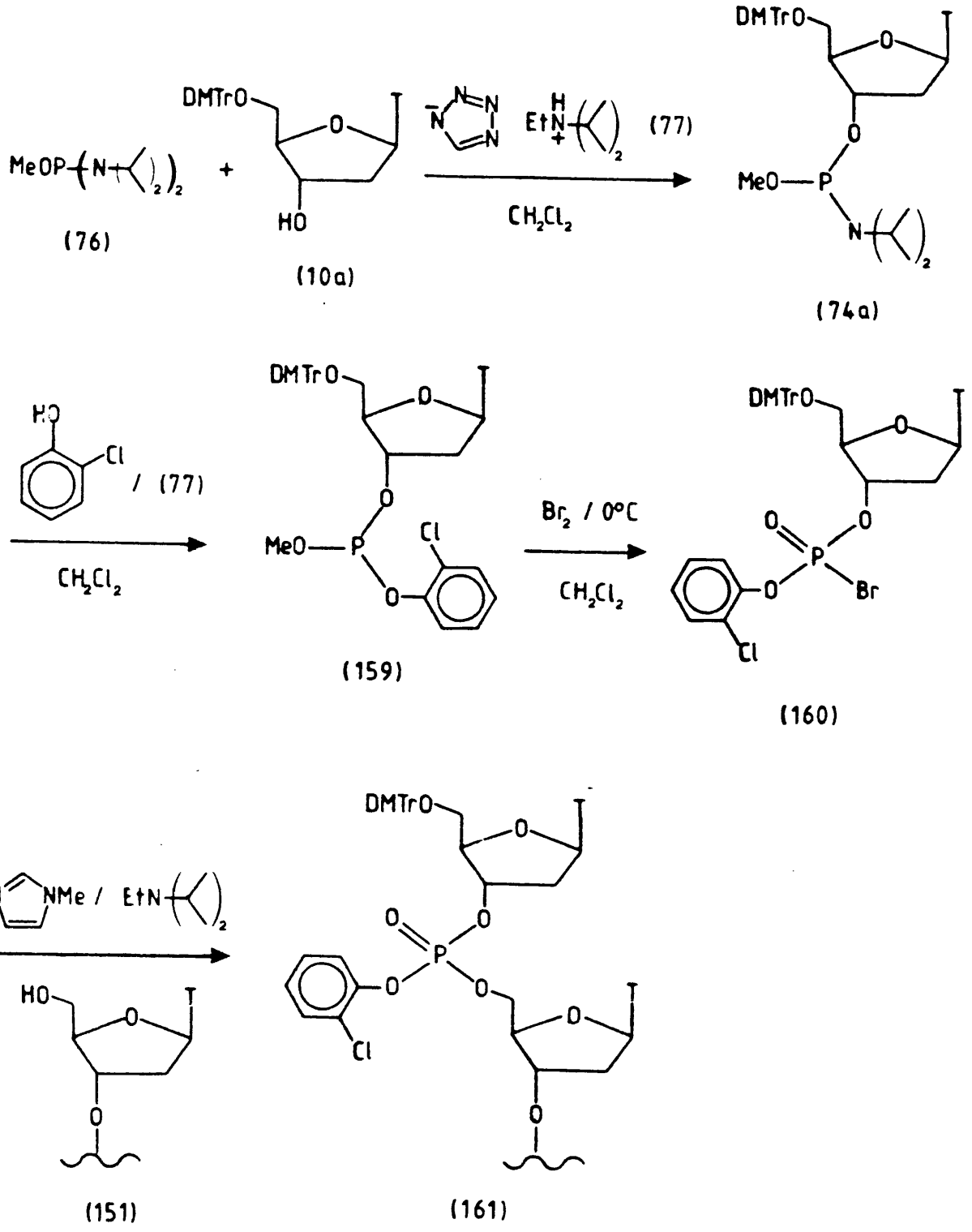
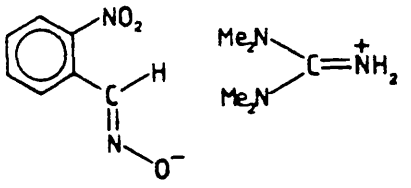


Figure 6.8

Following washing steps and terminal deprotections, stages that now included the N,N,N',N'-tetramethylguanidinium salt of syn-2-nitrobenzaldoxime (162), h.plc analysis, on reversed-phase indicated that, as before, poor phosphorylation had occurred, the majority of the mixture being due to unphosphorylated thymidine.



(162)

Time did not allow a thorough investigation of the 'alternative' approach shown in Figure 6.8, but it is suggested that this is the way ahead for the proposed Arbusov reaction approach. There is of course the problem of the exchange reaction that can occur once the phosphite (159) has been formed; which may account for the extra peak observed in the phosphite region of the ^{31}P n.m.r. spectrum. However, the exchange reactions investigated in Chapter 5 indicated that the o-chlorophenyl group did not exchange particularly rapidly but the fact that it eventually does means that a stock solution of the phosphite (159) would not be storable. Therefore, o-chlorophenyl would need to be added as required and followed by immediate treatment with bromine so as to keep the exchange reactions to a minimum.

These factors considered, therefore, suggest that the proposed Arbusov reaction route ought to be, eventually, workable.

Experimental

EXPERIMENTAL

General Experimental Details

M.p's were determined using a Kofler hot-stage apparatus and were uncorrected.

I.r. spectra were recorded with a Perkin-Elmer 298 instrument; maxima for P=O and C=O groups are quoted without assignment.

Electron-impact mass spectra were recorded with a V.G. Micromass 16B.

U.V. spectra were recorded with a Shimadzu UV-240 spectrophotometer.

Routine 'H n.m.r. spectra were recorded with a Varian EM390, 90 MHz spectrometer. High-field 'H n.m.r. spectra were recorded using a Bruker AM-300, 300 MHz spectrometer. For all 'H n.m.r. spectra tetramethylsilane was used as an internal standard and NH's and OH's were located by addition of D_2O.

Routine ^{31}P n.m.r. spectra ('H-decoupled) were recorded at 24.6 MHz with a JEOL JNM-FX-60 spectrometer; positive chemical shifts are downfield from external P^+

HPLC analysis were performed on a Shimadzu LC-4A liquid chromatograph fitted with a Whatman 25 cm x 4.6 mm Partisil-10 SAX column, for anion-exchange chromatography and Technicol 25cm x 4.6 mm Hypersil 50 ODS column for reversed-phase chromatography.

In general, reactions were conducted in an atmosphere of dry, oxygen free nitrogen.

Methanol was dried by refluxing over and distilling from magnesium turnings. Acetonitrile and methylene chloride were dried by refluxing over and distilling from calcium hydride and were stored over 3A molecular sieves. Diethyl ether was dried by refluxing over

and distilling from lithium aluminium hydride and stored over 3A molecular sieves. N,N-dimethylformamide was benzene azeotroped, stood over powdered barium oxide overnight and distilled under reduced pressure.[114] THF was dried by refluxing and distilling from sodium benzophenone ketal. Chloroform, hexane and ethanol were distilled prior to use. Pyridine (for general use) and triethylamine were dried by refluxing over and distilling from potassium hydroxide.

All nucleoside derivatives were dried by several coevaporations with anhydrous pyridine prior to use.

Tetrazole was sublimed using an Edwards High Vacuum System.

Ethanol was dried by refluxing over and distilling from magnesium turnings and isobutanol was dried by refluxing over and distilling from calcium hydride.

Preparation of Methyl Phosphorodichloridite (67)

This was prepared from phosphorus trichloride and methanol as described by Martin and Pizzolato.[133] Methanol was distilled from magnesium turnings and phosphorus trichloride was freshly distilled prior to use, δ ^{31}P n.m.r. (ether) +219.1 p.p.m. To phosphorus trichloride (34 g, 0.25 mol) at -78 ^{o}C (CO_2/acetone bath) was added slowly with vigorous stirring, methanol (8 g, 0.25 mol). The reaction is exothermic and releases copious amounts of hydrogen chloride gas. Upon completion of addition, 1 hour, the reaction mixture was allowed to attain room temperature and stirred at this temperature overnight. Fractional distillation, using a Vigreux column (300 mm) afforded methyl phosphorodichloridite as a colourless liquid (13.2 g, 39%). B.pt. 92-94 ^{o}C

δ ^{31}P n.m.r. (ether) + 180.3 p.p.m.

N.B. A yellow/orange precipitate forms upon continued distillation which may lead to instantaneous decomposition of the residue in the flask with inflammation. It is therefore recommended to cease the distillation when substantial amounts of this precipitate have been formed.

Attempted Preparation of t-Butyl Methyl Phosphorochloridite (83)

To a mixture of methyl phosphorodichloridite (7·9 g, 0.06 mol) and pyridine (4.7 g, 0.06 mol) in ether (250 cm^3) at -78 ^{o}C (CO_2/acetone bath) was added dropwise with stirring a solution of t-butanol (4.4 g, 0.06 mol) in the same solvent (50 cm^3, this solution at room temperature). Upon completion of addition (100 minutes) the reaction mixture was allowed to attain room temperature and stirred at this temperature for 20 minutes. The ^{31}P n.m.r. spectrum showed a major absorption at +164.9 p.p.m. due to (83) and a minor absorption at +130.3 p.p.m. assigned to di-t-butyl methyl phosphite. Pyridinium hydrochloride was separated by filtration

(celite-aided) under nitrogen and the solvent removed under reduced pressure to leave a cloudy oil (9.0 g). The ^{31}P n.m.r. spectrum of the crude mixture contained a major absorption at +3.0 p.p.m. with no absorption at +164.9 p.p.m. The peak at +3.0 p.p.m. was split into a doublet when the ^{31}P n.m.r. spectrum was recorded partially decoupled and the ^{1}H n.m.r. ($CDCl_3$) of the crude reaction mixture showed a doublet (J_{P-H} = 690 Hz). A ^{31}P n.m.r. chemical shift of +3.0 p.p.m. is consistent with literature values[81] for t-butyl methyl phosphite.

Preparation of Chloro-N,N-dimethylaminomethoxyphosphine (70)

This was prepared from dimethylamine and methyl phosphorodichloridite (67) as described by Caruthers[66]. To a solution of (67) (12.8 g, 0.096 mol) in ether (200 cm^3) at -78 °C (CO_2/acetone bath) was added dropwise with stirring dimethylamine (8.6 g, 0.19 mol) in the same solvent (50 cm^3). The addition was carried out over a period of 30 minutes after which the mixture was allowed to attain room temperature and was stirred at this temperature for 1 hour. Dimethylammonium hydrochloride was removed by filtration (celite-aided) and the solvent removed under reduced pressure to leave a cloudy oil (11.3 g). Distillation under reduced pressure gave chloro-N,N-dimethylaminomethoxyphosphine (70) as a colourless oil (9.2 g, 68%). B.pt. 38-40 °C, 12 mm/Hg (lit.[66] 40-42 °C, 13 mm/Hg).

δ ^{31}P n.m.r. (ether)	+177.6 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 2.6 (d., 6H, J_{P-H} = 12 Hz);
	3.6 (d., 3H, J_{P-H} = 15 Hz).

Preparation of t-Butyl Methyl N,N-dimethylphosphoramidite (85)

To a solution of chloro-N,N-dimethylaminomethoxyphosphine (5.3 g, 0.04 mol) in ether (150 cms^3) at -78 °C (CO_2/acetone bath) was added

dropwise with stirring a solution of t-butanol (2.6 g, 0.04 mol) and triethylamine (15.0 g, 0.15 mol) in the same solvent (100 cm^3) this solution at room temperature. Following addition (30 minutes) the solution was allowed to warm to room temperature and stirred at this temperature for a further 2½ hours. The triethylammonium hydrochloride was removed by filtration through a column of activated alumina (6 x 1 cm^3), the reaction flask being washed out well with ether. The solvent was removed under reduced pressure to leave a clear oil (3.7 g). Distillation of the crude reaction mixture under reduced pressure gave t-butyl methyl N,N-dimethylphosphoramidite (85) as a colourless oil (2.9 g, 40%). B.pt 26-28 ^oC, 7 mm/Hg.

δ ^{31}P n.m.r. (ether)	+139.4 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 1.3 (s., 9H); 2.55 (d., 6H, J_{P-H} = 9 Hz); 3.3 (d., 3H, J_{P-H} = 13 Hz)
ν max	2985; 2810; 2795; 1190; 1040; 980; and 975 cm^{-1}

Preparation of 5'-O-Dimethoxytritylthymidine (10a)

This was prepared according to the method of Gait.[134] To a solution of thymidine (4.6 g, 0.018 mol) in pyridine (15 cm^3) was added dimethoxytrityl chloride (7.1 g, 0.02 mol) and the mixture stirred overnight. Silica gel tlc analysis of the reaction mixture in chloroform:ethanol (93:7 v/v) showed one major trityl and uv positive spot at a higher R_f than the starting material. The pyridine was removed under reduced pressure to leave a gum which was triturated with benzene (10-15 cm^3) to leave a yellow/white powder which was filtered and dried (9.9 g). This was purified by column chromatography (silica gel using chloroform:ethanol: triethylamine, 92.5:7:0.5 as eluting solvent). Pure fractions were pooled,

dissolved in chloroform (4 cms^3) and precipitated into cold hexane (150 cm^3). The 5'-O-dimethoxytritylthymidine thus obtained was filtered off and dried (5.8 g, 56%).

'H n.m.r. ($CDCl_3$) δ 1.4 (s., 3H); 2.3 (m, 1H), 3.0 (m., 1H, 3.35 (m, 2H), 3.7 (s., 6H); 4.0 (m., 2H), 4.5 (m., 1H); 6.4 [t(b)]; 6.7 - 7.4 (m and AA' XX' pattern, 13H), 7.5 (s, 1H); 9.3 [s(b), 1H].

mass spec m/e M^+ 544, 303(100%), 302, 287, 273, 243, 227, 126.

Attempted Phosphitylation of (10a) with (85)

To a solution of t-butyl methyl N,N-dimethylphosphoramidite (0.16 g, 0.0009 mol) and 5'-dimethoxytritylthymidine (0.48 g, 0.0009 mol) in THF (10 cm^3) at room temperature was added by syringe a solution of tetrazole* (72), (0.07 g, 0.001 mol) in the same solvent (5 cm^3). After 25 minutes the ^{31}P n.m.r. spectrum of the crude reaction mixture showed two major absorptions at +134.9 p.p.m. due to the required phosphite (86) and at +3.4 p.p.m. Attempted isolation of the phosphite (86) by filtration through a small column of activated alumina and removal of solvent resulted in complete decomposition ; the ^{31}P n.m.r. spectrum containing two absorptions at +3.4 p.p.m. and +6.9 p.p.m. with no absorption in the phosphite region of the spectrum.

Preparation of t-Butyl Cyclopentyl Methyl Phosphite (84)

This compound was prepared by the method of Trippett.[76b] To a solution of methyl phosphorodichloridite (7.8 g, 0.06 mol) and pyridine

*Tetrazole (72) was purified by sublimation prior to use, this occurring at 110 °C, < 10^{-4} mbar.

(4.6 g, 0.06 mol) in ether (250 cm^3) at -78 °C (CO_2/acetone bath) was added dropwise with stirring a solution of t-butanol (4.3 g, 0.06 mol) in the same solvent (50 cm^3). The addition took place over a period of 100 minutes after which time a solution of pyridine (4.6 g, 0.06 mol) and cyclopentanol (5.02 g, 0.06 mol) in ether (50 cm^3) was added. Upon completion of addition (50 minutes) the cooling bath was removed and the mixture allowed to warm to room temperature and was stirred at this temperature for 20 minutes. The pyridinium hydrochloride was removed by filtration through a column of activated alumina (12 x 4 cm) and the reaction flask washed out well with ether. Following further elution of the column with ether (150 cms^3), the solvent was removed under reduced pressure to leave a clear oil (9.6 g). The ^{31}P n.m.r. spectrum of the crude reaction mixture showed a major absorption at +133.9 p.p.m., due to the required product and minor absorptions at +138.2 p.p.m. and +130.5 p.p.m., ascribed to <u>di</u>-t-butyl methyl phosphite and <u>di</u>-cyclopentyl methyl phosphite respectively. Distillation under reduced pressure afforded t-butyl cyclopentyl methyl phosphite as a colourless liquid. (6.4 g, 48%). B.pt. 80 °C, 5 mm/Hg.

δ ^{31}P n.m.r. (ether)	+133.9 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 1.4 (s., 9H); 1.75 (m., 8H); 3.34 (d., 3H, J_{P-H} = 13 Hz); 4.55(m., 1H)

<u>Preparation of Dimethyl 2-Methoxyethyl Phosphite (95)</u>

This was prepared by the method of Imaev.[88] A mixture of trimethyl phosphite (50.9 g, 0.41 mol), 2-methoxyethanol (48.4 g, 0.64 mol) and sodium (0.1 g) was heated under conditions for the distillative removal of methanol (b.pt. 64 °C) as it was formed at an oil bath temperature of 135 °C. When the methanol had ceased to distil , distillation under reduced pressure gave dimethyl 2-methoxyethyl phosphite as a colourless liquid,

(16.6 g, 25%). B.pt. 85 - 87 °C 12 mm/Hg.

δ ^{31}P n.m.r. (ether) +140.4 p.p.m.

^{1}H n.m.r. ($CDCl_3$) δ 3.38(s., 3H); 3.46(d., 6H, J_{P-H} = 13Hz);
3.63-3.99 (m, 4H).

Preparation of Dimethyl 2-Methoxyethyl Thiophosphate

This compound was prepared as described by Hoffmann and Moore.[135] To (2.5 g, 0.014 mol) of dimethyl 2-methoxyethyl phosphite at -78 °C, was added with stirring sulphur (0.95 g, 0.03 mol). The mixture was allowed to warm to room temperature and stirred at this temperature overnight. The excess sulphur was removed by filtration through a sintered glass funnel, the flask being washed out with ether. The solvent was removed under reduced pressure and small scale distillation, under reduced pressure afforded dimethyl 2-methoxyethyl thiophosphate as pale yellow liquid (0.99 g, 36%). B.pt. 65-70 °C 0.5 mm/Hg.

δ ^{31}P n.m.r. (CH_2Cl_2) +71.4 p.p.m.

^{1}H n.m.r. ($CDCl_3$) δ 3.33(s., 3H), 3.7(d., 6H, J_{P-H}=13 Hz)
4.0-4.3 (m., 4H).

ν max 2950, 2895, 2840, 1450,
1040 and 825 cm^{-1}

mass spec m/e M^{+} 200, 142, 141, 125, 78,
59(100%), 58, 28.

Preparation of 1-Methoxypropan-2-ol

This compound was prepared from propylene oxide and methanol in the presence of a catalytic amount of sodium hydroxide as described by Chitwood and Freure.[136] Purification by two fractional distillations gave

1-methoxypropan-2-ol (44.9 g, 45%). B.pt 116 °C. (lit[137] 118.3 °C).

^{1}H n.m.r. ($CDCl_3$) (lit[138])	δ 1.05(d., 3H, J = 6 Hz); 2.94(s., 1H, D_2O exchangeable); 3.1 - 3.2 (m., 2H); 3.3(s., 3H); 3.75 (m., 1H).

Preparation of Dimethyl 1-Methoxypropan-2-yl Phosphite (98)

This compound was prepared in the same manner as the phosphite (95) by the method of Imaev.[88] A mixture of trimethyl phosphite (62 g, 0.5 mol) 1-methoxypropan-2-ol (44.9 g, 0.5 mol) and sodium (0.05 g) was heated under conditions for the distillative removal of methanol (b.pt. 64 °C). Following this, distillation under reduced pressure gave dimethyl 1-methoxypropan-2-yl phosphite as a colourless liquid (33.2 g, 37%). B.pt. 74 - 77 °C 13mm/Hg.

δ ^{31}P n.m.r. (ether)	+140.4 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 1.2 (d., 3H, J = 6 Hz); 3.3 (s., 3H) 3.44 (d., 6H, J_{P-H} = 12 Hz); 3.7 (m, 2H., major d J = 11 Hz); 4.3 (m., 1H).

Preparation of Allyl Dimethyl Phosphite (100)

This compound was prepared in the same way as were the phosphites (95) and (98) from trimethyl phosphite (69 g, 0.56 mol), allyl alcohol (32.5 g, 0.56 mol) and sodium (0.2 g). Several fractional distillations under reduced pressure afforded allyl dimethyl phosphite as a colourless liquid (10.6 g, 13%)*. B.pt. 98 - 100 °C 60 mm/Hg.

δ ^{31}P n.m.r. (ether)	+140.6 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 3.45 (d., 6H, J_{P-H} = 13 Hz); 4.2-4.4 (m, 2H); 5.1-5.4 [m., 2H,

with resolvable dd J(trans) = 16 Hz,

J(gem) = ~ 1Hz]; 5.7-6.15 (m., 1H).

*The low yield is due to the fact that several fractional distillations were required to separate the required product from trimethyl phosphite and di-allyl methyl phosphite.

Preparation of 1-Chloro-2-methylpropan -2-ol

This compound was prepared from methallyl chloride as described by Burgin.[139] To methallyl chloride (252 g, 2.8 mol) in a one litre round bottomed flask at 5-10 °C was added with vigorous stirring 80% sulphuric acid (275 g, 2.8 mol). Upon completion of addition (45 minutes) the mixture was stirred for a further 2½ hours at 5-10 °C. Following this the mixture was poured in to ice and the mixture diluted to an acid strength of 10-15%. The required product distilled as a constant boiling liquid with water (b.pt. 96-100 °C, 835 g). To the cloudy distillate was added saturated sodium chloride solution (500 cm^3) which was then extracted with ether (2 x 250 cm^3). The ethereal layers were combined and dried ($MgSO_4$). Following filtration, the bulk of the ether was removed under reduced pressure and the residue distilled at atmospheric pressure to give 1-chloro-2-methylpropan-2-ol as a colourless liquid (104 g, 35%). B.pt. 124-126 °C (lit[139] 124-126 °C).

^1H n.m.r. ($CDCl_3$)	δ 1.3 (s, 6H), 3.15 (s, 1H, D_2O exchangeable), 3.4 (s, 2H).
ν max	3450, 2980, 1380, 1150, 910, 775 and 735 cm^{-1}.

Preparation of 2-Methylpropylene Oxide

This compound was prepared as described by Wilson and Lucas.[140] In a

one litre, three necked round bottomed flask (265 g, 4.7 mol) of potassium hydroxide pellets were dissolved in (135 g, 7.5 mol) of water with stirring. With the solution temperature at 70 ^{o}C, 1-chloro-2-methylpropan-2-ol (104 g, 0.96 mol) was added, with vigorous stirring, as fast as the rate of reflux would allow. Upon completion of addition (2½ hours) distillation at atmospheric pressure afforded 2-methylpropylene oxide as a colourless liquid (52.9 g, 77%). B.pt. 48-50 ^{o}C. (lit[141] 50.2-50.5 ^{o}C).

^{1}H n.m.r. ($CDCl_3$)	δ 1.25 (s., 6H); 2.39 (s, 2H).
ν max	2980, 2965, 1380, 1270, 1120 900 and 795 cm^{-1}.

Preparation of 1-Methoxy-2-methylpropan-2-ol

This compound was prepared in accord with the method of Chitwood and Freure.[136] To a refluxing solution of methanol (117 g, 3.7 mol) and sodium hydroxide (1 g) was added slowly with stirring 2-methylpropylene oxide (52 g, 0.73 mol) over a period of 3½ hours. Following addition the mixture was allowed to reflux for a further 2 hours. Rapid initial distillation removed the bulk of the excess methanol and subsequent fractional distillation afforded 1-methoxy-2-methylpropan-2-ol as a colourless liquid (53.7 g, 71%) B.pt. 114-117 ^{o}C. (lit[142] 117 ^{o}C).

^{1}H n.m.r. ($CDCl_3$)	δ 1.1 (s., 6H); 2.6 (s., 1H, D_2O exchangeable); 3.05 (s., 2H); 3.3 (s., 3H).
ν max	3450, 2970, 2920, 2880, 1155, 1115, 955 and 915 cm^{-1}.

Preparation of Cyclopentyl 1-Methoxy-2-methylpropan-2-yl Methyl Phosphite (103)

This compound was prepared using a similar procedure to that used for t-butyl cyclopentyl methyl phosphite (84). To a solution of methyl phosphorodichloridite (12.3 g, 0.093 mol) and pyridine (7.4 g, 0.093 mol) in ether (250 cm^3) at 0 ^oC (ice bath) was added dropwise with stirring a solution of 1-methoxy-2-methylpropan-2-ol (9.7 g, 0.093 mol) in the same solvent (50 cm^3). Upon completion of addition (45 minutes) the solution was allowed to stir for a further 45 minutes following which a solution of cyclopentanol (8g, 0.093 mol) and pyridine (7.4 g, 0.093 mol) in ether (50 cm^3) was added over a period of 45 minutes. Pyridinium hydrochloride was removed by filtration through a column of activated alumina (20 x 2 cms), the column being eluted well with ether. The solvent was removed under reduced pressure to leave a clear oil (21.4 g). Distillation under reduced pressure afforded cyclopentyl 1-methoxy-2-methylpropan-2-yl methyl phosphite as a cloudy oil (10.8 g, 47%). B.pt. 96-100 ^oC 0.4 mm/Hg.

δ ^{31}P n.m.r. (ether)	+132.9 p.p.m.*
^1H n.m.r. ($CDCl_3$)	δ 1.38 (s., 6H); 1.5-1.9 [m(b)., 8H]
	3.29 (s., 2H); 3.32 (s., 3H);
	3.4 (d., 3H, J_{P-H} = 13 Hz); 4.55 (m, 1H).

*The phosphite was contaminated with approximately 19% of a species at +129.3 p.p.m, assigned to di-1-methoxy-2-methylpropan-2-yl methyl phosphite.

Preparation of 1-Methylcyclopentanol

Approximately 5-10 cm^3 of a solution of methyl iodide (72.1 g, 0.5 mol) in ether was added to 12.4 g (0.5 mol) of magnesium turnings under anhydrous conditions in a one litre round bottom flask. Once reaction was underway (5-10 minutes) the rest of the methyl iodide/ether solution was added, with stirring, as fast as the rate of reflux would allow. Upon completion of addition (1¾ hours), the solution was refluxed for a further 1½ hours after which a solution of cyclopentanone (42.4 g, 0.5 moles) in ether (100 cm^3) was added as fast as the rate of reflux would allow. Following addition (1 hour), the mixture was refluxed for a further hour. The reaction was quenched by addition of 200 cm^3 of saturated ammonium chloride solution. The mixture was extracted with ether (2 x 200 cm^3) and the organic layers combined and dried ($MgSO_4$). Following removal of solvent under reduced pressure the crude product mixture was distilled under reduced pressure. The fraction collected (b.pt. 40°C, 20 mm/Hg) was shown not to be pure material, but was shown to contain unreacted cyclopentanone as was evident from the carbonyl stretching frequency in the infra red spectrum (1740 cm^{-1}). Pure material was obtained by sublimation (54 °C) yielding white needles of 1-methylcyclopentanol (8.7 g, 17%). M.pt. = 34-35 °C (lit[90] 36 °C).

^1H n.m.r. ($CDCl_3$)	δ 1.29 (s., 3H); 1.4-1.85 (m., 8H); 2.28(s., 1H, D_2O exchangeable)
ν max	3380, 2960, 1450, 1370, 1210, 1035, 940 and 905 cm^{-1}.

Preparation of Cyclopentyl 1-Methylcyclopentyl Methyl Phosphite (106)

This compound was prepared in the same way as was the phosphite (103). To a solution of methyl phosphorodichloridite (7.1 g, 0.053 mol) and pyridine (4.1 g, 0.053 mol) in ether (250 cm^3) at 0 ^oC (ice-bath) was added dropwise with stirring a solution of 1-methylcyclopentanol (5.3 g, 0.053 mol) in the same solvent (60 cm^3). Upon completion of addition (2 hours), the mixture was allowed to stir for a further 45 minutes after which a solution of cyclopentanol (4.3 g, 0.053 mol) and pyridine (4.1 g, 0.053 mol) in ether (50 cm^3) was added over a period of 2 hours. The ice bath was removed and the solution allowed to attain room temperature and was stirred at this temperature for a further 30 minutes. Pyridinium hydrochloride was removed by filtration through a column of activated alumina (2 x 20 cms), elution being carried out with ether (200 cm^3). The solvent was removed under reduced pressure to leave a cloudy oil (5.9 g).

δ ^{31}P n.m.r. (ether)	+134.3 p.p.m. (71%)
	minor peaks at +137.9 p.p.m. (18%)
	and at +131.3 p.p.m. (10%)
Crude ^1H n.m.r. ($CDCl_3$)	δ 1.48 (s., 3H); 1.5-2.25 (m., ~ 16H),
	3.35 (d., 3H, J_{P-H} = 13Hz);
	4.5 (m., 1H).

This compound was used without further purification since attempted distillation led to decomposition resulting in an absorption of +6.1 p.p.m. as the major component of the ^{31}P n.m.r. spectrum. This was shown from the ^1H n.m.r. spectrum to contain a P-H bond (J_{P-H} = 680 Hz). This phenomenom was also observed to a small extent with the preceding phosphite (103).

The Reaction of t-Butyl Cyclopentyl Methyl Phosphite (84) with Bromine

To a solution of t-butyl cyclopentyl methyl phosphite (0.77 g, 0.0035 mol) in deuterated methylene chloride (2 cm^3) at -78 °C (CO_2/acetone bath) was added bromine (0.56 g, 0.0035 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room remperature. The ^{31}P n.m.r. spectrum showed two phosphorus absorptions at +10.6 and -5.6 p.p.m. The ^1H n.m.r.spectrum of the solution was consistent with loss of t-butyl bromide in the Arbusov rearrangement due to the presence of a singlet at δ 1.8, consistent with literature[143] values for t-butyl bromide. No absorption was present at δ 2.6 characteristic of the proton in cyclopentyl bromide geminal to the bromine. The absorption at +10.6 p.p.m. in the ^{31}P n.m.r. spectrum was split into a doublet when the spectrum was recorded partially decoupled and the ^1H n.m.r. spectrum contained a doublet (J_{P-H} = 680 Hz) at δ 12.1 and δ 4.6.

The Reaction of Trimethyl Phosphite with Bromine

To a solution of trimethyl phosphite (0.56 g, 0.0045 mol) in deuterated methylene chloride (2 cm^3) at -78 °C (CO_2/acetone bath) was added bromine (0.72 g, 0.0045 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum contained one absorption at -4.03 p.p.m., characteristic of dimethyl phosphorobromidate (97).

^1H n.m.r. (CD_2Cl_2) δ 2.65 (s., 3H), δ 3,4 (d., 6H, J_{P-H} = 13 Hz).

The absorption at δ 2.65 is due to methyl bromide.

The Reaction of Dimethyl 2-Methoxyethyl Phosphite (95) with Bromine.

To a solution of dimethyl 2-methoxyethyl phosphite (0.59 g, 0.0035 mol) in deuterated methylene chloride (2 cm^3) at -78 °C (CO_2/acetone bath) was added bromine (0.53 g, 0.0035 mol) as a solution in the same solvent. The cooling bath was removed and the mixture allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture showed a major absorption at -5.9 p.p.m. and minor absorptions at -7.2 p.p.m. and +9.5 p.p.m. No absorption was present at -4.03 p.p.m. due to dimethyl phosphorobromidate (97). The ^1H n.m.r. spectrum of the solution was found to be consistent with the presence of equimolar amounts of methyl bromide and 2-methoxyethyl methyl phosphorobromidate (96).

^1H n.m.r. (CD_2Cl_2) δ 2.6 (s., 3H); 3.37 (s., 3H); 3.45-3.9 (m., 7H, containing a major doublet J_{P-H} = 13 Hz).

The Reaction of Dimethyl 1-Methoxypropan-2-yl Phosphite (98) with Bromine

To a solution of dimethyl 1-methoxypropan-2-yl phosphite (0.45 g, 0.0027 mol) in deuterated methylene chloride (2 cm^3) at -78 °C (CO_2/acetone bath) was added bromine (0.4 g, 0.0027 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting solution contained only one absorption at -6.7 p.p.m. The ^1H n.m.r. spectrum was consistent with loss of methyl bromide in the Arbusov reaction.

^1H n.m.r. (CD_2Cl_2) δ 1.4 (d., 3H, J = 6 Hz); 2.6 (s., 3H); 3.38 (s., 3H); 3.5 (m., 2H); 3.85 (d., 3H, J_{P-H} = 13 Hz); 4.8 (m., 1H).

The Reaction of Allyl Dimethyl Phosphite (100) with Bromine

To a solution of allyl dimethyl phosphite (0.25 g, 0.0017 mol) in carbon tetrachloride (2 cm^3) at -20 °C was added bromine (0.27 g, 0.0017 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture showed two absorptions at -4.03 p.p.m. and -5.9 p.p.m in the ratio of 2:1 (by ^{31}P n.m.r.). The absorption at -4.03 p.p.m. indicated that dimethyl phosphorobromidate (97) had been formed. The ratios indicated a 2:1 preference for loss of the allyl substituent, statistically 4:1 as there are two methyl groups. However, some methyl bromide was produced as was evident from a singlet at δ 2.6 in the ^1H n.m.r. spectrum.

The Reaction of Cyclopentyl 1-Methoxy-2-methylpropan-2-yl Methyl Phosphite (103) with Bromine

To a solution of (103, 1.2 g, 0.0049 mol) in carbon tetrachloride (2 cm^3) at -20 °C was added bromine (0.78 g, 0.0049 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture was quite complex although the major absorptions occurred at +6.7 p.p.m. and -7.7 p.p.m. Minor absorptions were present at +8.7 p.p.m., +4.2 p.p.m. and +3.0 p.p.m. The peak at +6.7 p.p.m. was shown to contain a P-H bond as it was split into a doublet when the spectrum was recorded partially decoupled. One half of the doublet (J_{P-H} = ~680 Hz) was visible at δ 11.8 in the ^1H n.m.r. spectrum.

The Reaction of (103) with Bromine in the presence of Proton Sponge

To a solution of (103, 0.18 g, 0.00072 mol) and 1,8-bisdimethylaminonaphthalene (0.62 g, 0.0029 mol) in carbon tetrachloride (3 cm^3) at 0 ^oC (ice-bath) was added bromine (0.12 g, 0.00075 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture showed one major absorption at -7.9 p.p.m. with minor absorptions at +6.5 p.p.m, +4.0 p.p.m. and -12.7 p.p.m. The ^1H n.m.r. (CCl_4), although complex, contained a singlet at δ 1.7 characteristic of the methyl groups in 2-bromo-2-methyl-3-methoxypropane, the alkyl halide that would result from loss of the tertiary substituent in the Arbusov reaction. In addition, no absorption was present at δ 2.6 due to methyl bromide, nor at δ 4.1 due to the proton in cyclopentyl bromide geminal to the bromine.

The Reaction of Cyclopentyl 1-Methylcyclopentyl Methyl Phosphite (106) with Bromine

To a solution of (105, 0.62 g, 0.0025 mol) in carbon tetrachloride (2 cm^3) at -20 ^oC, was added bromine (0.4 g, 0.0025 mol) as a solution in the same solvent. The cooling bath was removed and the mixture allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture contained a major absorption at +7.1 p.p.m. and a minor absorption at -7.7 p.p.m, similar to the result obtained with the phosphite (103). The ^1H n.m.r. spectrum contained no absorptions due to methyl bromide nor cyclopentyl bromide but an additional singlet at δ 2.0 which may be due to the methyl group alpha to the bromine in 1-bromo-1-methylcyclopentane. The peak at +7.1 p.p.m. was split into a doublet when the spectrum was recorded partially decoupled, a feature

which was also prominent in the ^1H n.m.r. spectrum at δ 10.5 and δ 2.8(J_{P-H} = ~ 680 Hz).

The Reaction of (106) with Br mine in the presence of Proton Sponge.

To a solution of (106, 0.43 g, 0.0017 mol) and 1,8-bis-dimethylaminonaphthalene (0.37 g, 0.0018 mol) in carbon tetrachloride (3 cm^3) at -20 °C was added bromine (0.28 g, 0.0017 mol) as a solution in the same solvent. Following the removal of the cooling bath the solution was allowed to warm to room temperature. The ^{31}P n.m.r. spectrum showed the major absorption to be at -7.9 p.p.m., due, presumably to cyclopentyl methyl phosphorobromidate (93), with minor absorptions at +7.1 p.p.m., -10.9 p.p.m. and -12.1 p.p.m. Upon standing overnight the peak due to the bromidate (93) at -7.9 p.p.m. had become the minor component of the mixture, the major components being at -10.7 p.p.m., -12.1 p.p.m., -13.7 p.p.m. and -14.3 p.p.m.

The Reaction of (106) with Bromine in the presence of Proton Sponge. in Acetonitrile

To a solution of (106, 0.36 g, 0.0015 mol), 1,8-bisdimethyl-aminonaphthalene (0.33 g, 0.0015 mol) in acetonitrile (4 cm^3) at -35 °C was added bromine (0.25 g, 0.0015 mol), as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum of the resulting solution showed one major absorption at -8.1 p.p.m. with minor absorptions at -11.5 p.p.m., -12.7 p.p.m. and +8.1 p.p.m. Upon standing overnight, the peak at -8.1 p.p.m. became a minor component of the mixture, the major absorptions being at -1.4 p.p.m., -11.5 p.p.m. and -12.7 p.p.m.

The Preparation of Dimethyl Phosphorochloridite (109).

This compound was prepared by an adaptation of the method of Ramirez[92]. To phosphorus trichloride (34.2 g, 0.25 mol) at 0 °C (ice-bath) was added, dropwise with stirring, trimethyl phosphite (76.6 g, 0.56 mol) over a period of 45 minutes. Upon completion of addition the cooling bath was removed and the solution allowed to attain room temperature and was stirred at this temperature overnight. Two fractional distillations under reduced pressure (b.pt. 32-35°C 35 mm/Hg) and one at atmospheric pressure through a column of glass helices (16 x 2 cm) afforded dimethyl phosphorochloridite as a colourless liquid (10.6 g, 11%). B.pt. 96-100 °C (lit[92] 96-108 °C).

δ ^{31}P n.m.r. (ether)	+169.9 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 3.62 (d., 3H, J_{P-H} = 11 Hz)

N.B. The precautions recommended when distilling methyl phosphorodichloridite (67) are also recommended in the distillation of dimethyl phosphorochloridite (109).

The Preparation of 5'-O-Tritylthymidine (15)

This was prepared in the same way as was 5'-O-dimethoxytritylthymidine (10a). To a solution of thymidine (10.4 g, 0.043 mol) in pyridine (50 cm^3) was added trityl chloride (14.4 g, 0.054 mol). The mixture was stirred at room temperature overnight. Silica gel tlc analysis of the reaction mixture in chloroform:ethanol (86:14 v/v) showed one major trityl and uv positive spot at a higher R_f than the starting material. The pyridine was removed under reduced pressure to leave a gum (32.8 g) which was dissolved in methylene chloride (150 cm^3) and washed thoroughly with water (4 x 100 cm^3) and dried

(Na_2SO_4). Following filtration, the solvent was removed under reduced pressure to leave a gum (16.4 g) which was purified by column chromatography (silica gel using chloroform : ethanol 93:7 v/v as eluting solvent). Pure fractions were pooled, dissolved in methylene chloride (8 cm^3) and precipitated into cold hexane (250 cm^3). The 5'-O-tritylthymidine obtained was filtered off and dried (15.1 g, 73%). Tlc analysis on silica gel using chloroform : ethanol (86:14) showed one spot with an R_f of 0.55, which was identical to that of an authentic sample of (15). The 'H n.m.r. was also found to be identical to that of an authentic sample.

'H n.m.r. ($CDCl_3$) δ 1.5 (s., 3H); 1.9 (s., 1H, D_2O exchangeable); 2.3-2.45 (m., 2H), 3.35 - 3.45 (m., 2H); 4.0 (m., 1H); 4.5 (m., 1H); 6.35 [t(b)., 1H]; 7.2-7.4 (m, 15H, aromatics) 7.5 [s(b), 1H]; 8.5 (s., 1H, D_2O exchangeable).

mass spec m/e M^+ 484, 242, 241, 165, 126, 105, 77(100%).

The Prepartion of 5'-O-Tritylthymidine-3'-O-(isobutyl methyl phosphate)(117)

To a solution of dimethyl phosphorochloridite (0.19 g, 0.0015 mol) in methylene chloride (1 cm^3) at -78 °C (CO_2/acetone bath) was added with stirring a solution of 5'-O-tritylthymidine (0.75 g, 0.0015 mol) and diisopropylethylamine (0.2 g, 0.0015 mol) in the same solvent (2 cm^3). The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum contained a major absorption at +140.3 p.p.m. due to the dimethyl nucleoside phosphite (115) and a minor absorption at +10.7 p.p.m. due to dimethyl phosphite (lit[143] δ ^{31}P n.m.r. +11± 1 p.p.m.). The solution was cooled again to -78 °C and bromine (0.25 g, 0.0015 mol) was added as a solution in

methylene chloride. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum contained two absorptions of equal intensity at +4.5 p.p.m. and +4.4 p.p.m. due to the two diastereoisomers of the phosphorobromidate (116). To this solution, now at room temperature was added a mixture of isobutanol (0.12 g, 0.0015 mol), diisopropylethylamine (0.2 g, 0.0015 mol) and 1-methylimidazole (0.51 g, 0.0061 mol). The ^{31}P n.m.r. spectrum recorded within five minutes showed a major absorption at -0.6 p.p.m. with two minor absorptions at +11.1 p.p.m. and -12.3 p.p.m.

The reaction mixture was poured into a 250 cm^3 separating funnel, the reaction flask being washed out with methylene chloride. This was washed with saturated sodium thiosulphate solution (2 x 100 cm^3) and then with water (2 x 100 cm^3) before being dried over Na_2SO_4. The drying agent was removed by filtration and the solvent removed under reduced pressure to leave a brown foam (0.68 g, 73% crude recovery). The ^{31}P n.m.r. spectrum contained one absorption at -0.6 p.p.m. The crude material was purified by column chromatography (silica gel using chloroform : ethanol 97:3 v/v as eluting solvent). Pure fractions (R_f = 0.24) were pooled, dissolved in chloroform (1 cm^3) and precipitated into cold hexane (100 cm^3). The white powder of 5'-O-tritylthymidine-3'-O-(isobutyl methyl phosphate) (117) thus obtained was filtered and dried (0.32 g, 34%).

δ ^{31}P n.m.r.	-0.6 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 0.8-1.0 (2 x d., 6H, J = 8Hz);
	1.4(s., 3H); 1.7-2.1(m., 1H) 2.3-2.6(m., 2H);
	3.35-3.5(m., 2H);
	3.55-3.85(m., 5H); 4.25[s(b)., 1H];
	5.05-5.25(m., 1H);
	6.4-6.55 [t(b)., 1H]
	7.1-7.45 (m., 15H, aromatics);
	7.5(s., 1H); 9.8(s., 1H).

The Preparation of Dimethyl Isobutyl Phosphate (118)

To a solution of trimethyl phosphite (1.5 g, 0.012 mol) and diisopropylethylamine (1.5 g, 0.012 mol) in acetonitrile (2 cm^3) at -30 ^oC was added bromine (1.9 g, 0.012 mol) as a solution in the same solvent. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum contained one absorption at -4.4 p.p.m. due to dimethyl phosphorobromidate (97). To this solution was then added isobutanol (0.9 g, 0.012 mol) and 1-methylimidazole (4.9 g, 0.06 mol). The ^{31}P n.m.r. spectrum recorded within five minutes showed three absorptions at +1.0 p.p.m., -12.5 p.p.m and -1.2 p.p.m.

The reaction mixture was poured into a 250 cm^3 separating funnel, the reaction flask being washed out well with methylene chloride. The organic extract was washed with dilute HCl solution (2 x 60 cm^3) and then with water (2 x 60 cm^3) before being dried over $MgSO_4$. Following filtration, the solvent was removed under reduced pressure to leave a dark red oil (2.1 g). Small scale distillation under reduced pressure afforded dimethyl isobutyl phosphate as a light brown coloured liquid (0.87 g, 40%. B.pt. 46-48 ^oC 0.1mm/Hg.)

δ ^{31}P n.m.r. (CH_2Cl_2)	+1.4 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 0.9 (d., 6H, J = 8Hz); 1.75-2.1(m., 1H); 3.55-3.83 (m., 8H with resolved d., J_{P-H} = 12Hz).
ν max	2980, 1275, 1040 and 850 cm^{-1}.

The Preparation of 5'-O-Tritylthymidine-3'-O-(3'-O-acetylthymidyl methyl phosphate) (119)

To a solution of dimethyl phosphorochloridite (0.13 g, 0.001 mol)

in methylene chloride (1 cm^3) at room temperature was added dropwise a solution of 5'-O-tritylthymidine (0.5 g, 0.001 mol) and diisopropylethylamine in the same solvent (2 cm^3). The ^{31}P n.m.r. spectrum of the reaction mixture contained a major absoprtion at +140.6 p.p.m. due to the required phosphite and a minor absorption at +10.9 p.p.m. due to dimethyl phosphite. The solution was cooled to -78 ^oC (CO_2/acetone bath) and one molar equivalent of bromine (0.16 g, 0.001 mol) as a solution in methylene chloride was added. The cooling bath was removed and the solution allowed to warm to room temperature. The ^{31}P n.m.r. spectrum contained a major absorption at +4.6 p.p.m. due to the required phosphorobromidate (the diastereoisomers on this occasion were not resolved). To this solution was now added 3'-O-acetylthymidine (0.39 g, 0.0014 mol), 1-methylimidazole (0.46, 0.0056 mol) and diisopropylethylamine (0.18 g, 0.0014 mol) in methylene chloride (2 cm^3). The ^{31}P n.m.r. spectrum recorded within five minutes showed the major absorption to be at -12.5 p.p.m. with minor absorptions at -0.8 p.p.m. and -1.4 p.p.m.

The reaction mixture was poured into a 250 cm^3 separating funnel, the reaction being washed out well with methylene chloride. The organic phase was washed with saturated sodium thiosulphate solution (2 x 50 cm^3) and then with water (2 x 50 cm^3) and then dried over Na_2SO_4. The drying agent was filtered off and the solvent removed under reduced pressure to leave a brown foam (0.6 g, 72% crude recovery). Purification was carried out by column chromatography (silica gel using chloroform : ethanol as the eluting solvent). Pure fractions (R_f = 0.33) were pooled, dissolved in chloroform (1 cm^3) and precipitated into cold hexane to give a white powder which was filtered and dried (0.19 g, 24%) and was shown by n.m.r. to be the required phosphate (119).

δ ^{31}P n.m.r. (CH_2Cl_2) -0.4 p.p.m.

^{1}H n.m.r. ($CDCl_3$) δ1.35-1.45 (2 x s., 6H); 2.0(s., 3H); 2.15-2.6(m., 4H); 3.3-3.45(m., 2H); 3.55-3.8(m., 5H, two slightly resolveable doublets J_{P-H} = 11Hz); 4.0(m., 3H); 4.5 (m., 1H); 5.05-5.3(m., 1H); 6.2-6.4[t(b), 2H]; 7.05-7.4(m., 15H, aromatics); 7.5(s, 2H); 9.3-9.5[s(b), 2H].

The preparation of 5'-O-Dimethoxytritylthymidine-3'-O-(isobutyl methyl phosphate) (112a).

To a solution of dimethyl phosphorochloridite (0.21 g, 0.0016 mol) in methylene chloride (2 cm^3) at room temperature was added a solution of 5'-O-dimethoxytritylthymidine (0.89 g, 0.0016 mol) and diisopropylethylamine (0.84 g, 0.065 mol) in the same solvent (2 cm^3). The ^{31}P n.m.r. spectrum showed the major absorption to be present at +140.6 p.p.m. and a minor absorption at +10.7 p.p.m. The solution was cooled to -78 ^oC (CO_2/acetone bath) and one molar equivalent of bromine (0.26 g, 0.0016 mol) was added to a solution in the same solvent followed by a solution of isobutanol (0.12 g, 0.0016 mol) and 1-methylimidazole (0.54 g, 0.065 mol) in methylene chloride (2 cm^3). The cooling bath was removed and the solution allowed to warm to room temperature. The reaction mixture was worked-up as before and the solvent removed under reduced pressure to leave a brown gum (2g). Purification was effected by column chromatography (silica gel using chloroform : ethanol : triethylamine 97.5:1.5:1 v/v as eluting solvent). Pure fractions (R_f = 0.2) were pooled, dissolved in chloroform (1 cm^3) and precipitated into cold hexane to give a white powder which was filtered and dried (0.38 g, 34%).

δ ^{31}P n.m.r. (CH_2Cl_2) -0.6 p.p.m.

'H n.m.r. ($CDCl_3$) δ0.8-1.0 (2 x d, 6H, J = 8Hz); 1.45(s., 3H); 1.7-2.0(m., 1H); 2.3-2.7(m., 2H); 3.32-3.5 (m., 2H); 3.57-3.9(m., 11H, including prominent s 6H); 4.18-4.32 (m, 1H); 5.05-5.3 (m, 1H); 6.3-6.5 [t(b), 1H]; 6.7-7.4 (m, 13H, including AA'XX' pattern, aromatics); 7.5 [s(b), 1H], 8.7-8.8[s(b), 1H].

The Preparation of 5'-O-Dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine 3'-O-(isobutyl methyl phosphate) (112b).

This compound was prepared in an identical manner to the preceding phosphate (112a), beginning from dimethyl phosphorochloridite (0.1 g, 0.00079 mol) and 5'-O-dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine (0.49 g, 0.00079 mol). The required phosphate (112b), following chromatography and precipitation into hexane was isolated as a white powder (0.15 g, 25%).

δ ^{31}P n.m.r. (CH_2Cl_2) -0.8 p.p.m.

'H n.m.r. ($CDCl_3$) δ 0.75-0.95 (2 x d, 6H, J = 8Hz); 1.7-1.9 (m, 1H); 2.2-2.6 (m, 2H); 3.33-3.5 (m, 2H); 3.53-3.8 (m, 11H, prominent s, 6H); 4.2-4.4 (m, 1H); 4.8-5.16 [m(b), 1H]; 6.1-6.3 [t(b), 1H]; 6.65-7.33 (m, 18H, including AA'XX' pattern, aromatics); 7.45 [s(b), 1H]; 7.7-7.9 (m, 1H); 8.0-8.15 (m, 1H).

The major impurity (by ^{31}P n.m.r.) had a chemical shift of -1.2 p.p.m. and was hitherto unidentified.

The Preparation of 5'-O-Dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine 3'-O-(isobutyl methyl phosphate) (112c).

This compound was prepared in the same way as before from dimethyl phosphorochloridite (0.087 g, 0.00068 mol) and 5'-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine (0.44 g, 0.000068 mol). The required phosphate (112c) was isolated as a white powder following chromatography and precipitation into cold hexane (0.094 g, 17%).

δ ^{31}P n.m.r. (CH_2Cl_2)	-0.8 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 0.8-1.0 (2 x d, 6H, J = 8Hz);
	1.8-2.25 (m, 1H), 2.9 (m, 2H);
	3.3-3.45 [d(b), 2H, J = ~ 5Hz];
	3.62-3.83 (m, 11H, prominent s 6H)
	4.3-4.5 (m, 1H); 5.1-5.3 [m(b), 1H];
	6.35-6.68 [t(b), 1H]; 6.74-7.32 (m, 18H,
	including AA'XX' pattern, aromatics);
	7.85-8.0 (m, unidentified); 8.1 (s, 1H);
	8.6 (s, 1H); 9.05 [s(b), 1H].

The polarity of the eluting solvent was increased to 84 : 14.5 : 0.5, v/v chloroform : ethanol : triethylamine following elution of the required phosphate so as to elute from the column a more polar component of the mixture which was shown to be 5'-O-dimethoxytrityl-N^6-benzoyl-2'-adenosine-3'-O-(methyl phosphate). (120a) (0.24 g, 47%).

δ ^{31}P n.m.r. (CH_2Cl_2)	+0.4 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 1.2-1.4 (t, 9H, J = ~ 6Hz);
	2.3-2.75 (m, 2H); 2.8-3.2 (q, 6H, J =~ 6Hz);
	3.34-3.5(m, 2H); 3.55 (d, 3H, J_{P-H} = 12Hz);
	3.7 (s, 6H); 4.33-4.55 (m, 1H); 4.86-5.18
	[m(b), 1H]; 6.5-6.6 [t(b), 1H];

6.61-7.45 (m, 18H, including AA' XX' pattern, aromatics); 7.8-8.05 (m, unidentified); 8.15(s, 1H); 8.6 (s, 1H); 9.2-9.55 [s(vb), 1H].

The Preparation of 5'-O-Dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine-3'-O-(isobutyl methyl phosphate) (112d).

This compound was prepared as before beginning from dimethyl phosphorochloridite (0.083 g, 0.00065 mol) and 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine (0.41 g, 0.00065 mol). Following column chromatography the product was not sufficiently pure. Therefore, subsequent preparative thin layer chromatography was carried out on silica gel using chloroform : ethanol : triethylamine (98:1.5:0.5 v/v) as eluting solvent. Following elution of the preparative plate, the required band was scraped off and extracted with methanol : ether 25 cm^3 (50 : 50 v/v) for 30 minutes. Following this the silica was filtered off (washed well with ether : methanol) and the solvent removed under reduced pressure to leave an oil which was dissolved in chloroform (1 cm^3) and precipitated into a cold hexane to give a white powder of (112d) (0.1 g, 20%).

δ ^{31}P n.m.r. (CH_2Cl_2) -0.4 p.p.m.

^1H n.m.r. ($CDCl_3$) δ 0.8-0.95 (2 x d, 6H, J = 8 Hz); 1.0-1.12 (2 x d, 6H, J = 7 Hz); 2.4-2.8 (m, 2H); 3.2-3.4 (m, 2H); 3.55-3.85 (m, 11H, prominent s, 6H); 4.1-4.3 (m, 1H); 5.42-5.7 (m, 1H); 6.01-6.3 [t(b), 1H]; 6.63-7.48 (m, 14H, including AA'XX' pattern, aromatics); 7.63 (s, 1H).

The Preparation of 5'-O-Dimethoxytritylthymidine-3'-O-(isobutyl methyl phosphate) (112a) under optimum conditions.

To a solution of dimethyl phosphorochloridite (0.24 g, 0.0019 mol) in methylene chloride (1 cm^3) was added dropwise with stirring via a teflon cannula a solution of 5'-O-dimethoxytritylthymidine (1 g, 0.0019 mol) and diisopropylethylamine (0.25 g, 0.0019 mol) in the same solvent (3 cm^3). The ^{31}P n.m.r. spectrum recorded immediately showed one phosphorus species at +140.4 p.p.m. [figure 3.13(i), page 80]. The solution was cooled to 0 ^oC (ice-bath) and one molar equivalent of bromine was added (0.3 g, 0.0019 mol) as a solution in the same solvent. The ^{31}P n.m.r. spectrum [Figure 3.13(ii) and (iii)] showed one phosphorus species at +4.6 p.p.m. (two peaks separated by 5.4 Hz when the spectrum was recorded on a 1 KHz sweep width) due to the required phosphorobromidate (110a). The cooling bath was removed and the solution allowed to warm to room temperature whereupon a solution of isobutanol (0.14 g, 0.0019 mol), 1-methylimidazole (0.16 g, 0.0019 mol) and diisopropylethylamine (0.25 g, 0.0019 mol) in methylene chloride (4 cm^3) was added. The ^{31}P n.m.r. spectrum recorded immediately showed one absorption at -0.8 p.p.m.

The dark coloured reaction mixture was poured into a 250 cm^3 separating funnel, the reaction flask being washed out well with methylene chloride. The organic phase was washed with one molar sodium thiosulphate solution (100 cm^3), one molar sodium bicarbonate solution (100 cm^3) and water (2 x 100 cm^3) before being dried over Na_2SO_4. The drying agent was filtered off and the solvent removed under reduced pressure to leave a brown foam (1.7 g) which was purified by column chromatography (silica gel using chlorofrom : ethanol : triethylamine, 97:2:1 v/v as eluting solvent). Pure fractions (R_f = 0.2) were pooled, dissolved in chloroform (2 cm^3) and precipitated into cold hexane (150 cm^3). The white powder of (112a) was filtered and dried (0.83 g, 69%).

The Reaction of Diethyl Phosphorobromidate (123) with Cyclopentanol.

In each reaction described, prior to the phosphorylation of cyclopentanol, diethyl phosphorobromidate was generated "in-situ" from triethyl phosphite and bromine.

(i) In the Presence of Diisopropylethylamine

To a solution of triethyl phosphite (0.94 g, 0.0056 mol) in acetonitrile (5 cm^3) at 0 ^oC (ice-bath) was added dropwise with stirring a solution of bromine (0.9 g, 0.0056 mol) in the same solvent (5 cm^3). The ^{31}P n.m.r. spectrum showed exclusive conversion of triethyl phosphite (δ ^{31}P n.m.r. (CH_3CN) = +138.4 p.p.m.) into diethyl phosphorobromidate (δ ^{31}P n.m.r. (CH_3CN) =-8.7 p.p.m., lit[75] δ ^{31}P n.m.r. (CCl_4) = -8.9 p.p.m.).

The cooling bath was removed and a solution of cyclopentanol (0.48 g, 0.0056 mol) and diisopropylethylamine (1.5 g, 0.011 mol) in acetonitrile (5 cm^3) was added. The subsequent reaction was monitored by ^{31}P n.m.r. spectroscopy. The reaction was left overnight, it taking 16 hours in total for the signal at -8.7 p.p.m. to be replaced by two absorptions at -2.2 p.p.m. and -13.3 p.p.m., present as 66% and 43% of the mixture resepctively.

The solution was poured into a 250 cm^3 separating funnel, the reaction flask being washed out well with methylene chloride. The organic phase was washed with dilute HCl solution (2 x 100 cm^3) and then with water (2 x 100 cm^3) before being dried over Na_2SO_4. Following filtration the solvent was removed under reduced pressure to leave a brown oil (0.8 g, 64% crude recovery). Small scale distillation under reduced pressure afforded cyclopentyl diethyl phosphate (124) as a colourless liquid (0.49 g, 40%). B.pt. 76-78 ^oC. 0.1 mm/Hg.

δ ^{31}P n.m.r. (CH_2Cl_2)	-2.2 p.p.m.
^1H n.m.r.	δ 1.2-1.4 (t, 6H, J = 7Hz);

1.5-1.95 [m(b), 9H];

3.86-4.2 (dq J_{P-H} ~ J_{H-H} ~ 7Hz);

4.75-4.95 [m(vb), 1H].

ν max 2975, 1270, 1160, 1030 (vb) and 835 cm^{-1}.

mass spec m/e M^+ 222, 125 (100%), 99, 33, 29.

(ii) In the Presence of N,N-Dimethylformamide (DMF).

Prior to use, DMF was dried by the method of Thomas and Rochow.[114] Diethyl phosphorobromidate (123) was generated as before from triethyl phosphite (1.57 g, 0.0095 mol) and bromine (1.5 g, 0.0095 mol). To the solution of (123) at room temperature was added dropwise with stirring, a solution of diisopropylethylamine (2.5 g, 0.019 mol) and DMF (1.4 g, 0.019 mol). The ^{31}P n.m.r. spectrum showed no change in the chemical shift of (123) from -8.7 p.p.m. although a minor absorption due to tetraethyl pyrophosphate [125, δ ^{31}P n.m.r. (CH_3CN) = -13.7 p.p.m.] was present. To this solution was then added cyclopentanol (0.82, 0.0095 mol) in acetonitrile (5 cm^3). The ^{31}P n.m.r. spectrum recorded immediately showed no absorption due to (123) but two absorptions at -2.2 p.p.m. due to the required product (124) and at -13.5 p.p.m. due to tetraethyl pyrophosphate (125). The intensities of the two absorptions were 52% and 48% respectively, by ^{31}P n.m.r.

(iii) In the Presence of N,N'-dimethyl-N,N'-propylene Urea (DMPU, 128).

Prior to use DMPU was dried by distillation under reduced pressure from calcium hydride, b.pt. 118-120 ^oC 1.5 mm/Hg. Diethyl phosphorobromidate (123) was generated from triethyl phosphite (1.2 g, 0.0074 mol) and bromine (1.18 g, 0.0074 mol), as before. To the solution of (123) in

acetonitrile (6 cm^3) at room temperature was added dropwise with stirring a solution of diisopropylethylamine (1.9 g, 0.015 mol), DMPU (1.02 g, 0.0079 mol) and cyclopentanol (0.64 g, 0.0074 mol) in the same solvent (2 cm^3). The ^{31}P n.m.r. spectra of the subsequent transformations were monitored after 5 minutes, 30 minutes, 60 minutes and 4 hours after which time the absorption due to (123) had been replaced by several phosphorus containing species, the most prominent of which was due to the required product. The reaction had proceeded through an apparent intermediate as was evident by the growth of an absorption at -9.9 p.p.m. in the ^{31}P n.m.r. spectrum and its subsequent disappearance (Figure 4.5) at the expense of the product peak. Following work-up as described in the uncatalysed reaction the product (124) was isolated by small scale vacuum distillation in 25% yield.

(iv) In The Presence of 1-Methylimidazole (51)

1-Methylimidazole was distilled under reduced pressure prior to use, b.pt. 64-66 ^{o}C 0.35 mm/Hg.

To a solution of diethyl phosphorobromidate [123 , generated from triethyl phosphite (1.45 g, 0.0088 mol) and bromine (1.4 g, 0.0088 mol)] in acetonitrile(10 cm^3) at room temperature was added dropwise with stirring a solution of diisopropylethylamine (1.13 g, 0.0088 mol), 1-methylimidazole (3.6 g, 0.04 mol) and cyclopentanol (0.76 g, 0.0088 mol). The ^{31}P n.m.r. spectrum, recorded within five minutes showed the absorption due to (123) to have been replaced by two absorptions at -2.2 p.p.m. (75%) and -11.5 p.p.m. (25%). The reaction was worked-up as before and cyclopentyl diethyl phosphate (124) was isolated in 48% yield by distillation.

The Reaction of Diethyl Phosphorobromidate with Imidazole

To a solution of diethyl phosphorobromidate [generated from triethyl phosphite (2 g, 0.012 mol) and bromine (1.93 g, 0.012 mol)] in acetronitrile

(5 cm^3) was added, by syringe, a solution of imidazole (1.64 g, 0.024 mol) in the same solvent (15 cm^3). The ^{31}P n.m.r. spectrum recorded after 10 minutes contained a major absorption at -6.24 p.p.m. presumably due to the diethyl phosphoro-imidazolium species (133, Figure 4.9, page 95). Attempted isolation, including aqueous washings resulted in degradation, the major absorption in the ^{31}P n.m.r. being at -13.5 p.p.m. due to tetraethyl pyrophosphate, and minor absorptions at +1.4 p.p.m. and +7.3 p.p.m.

The Preparation of Phenyl Phosphorodichloridite (140)

This compound was prepared by the method of Tolkmith.[144] To neat phosphorus trichloride (173.8 g, 1.26 mol) maintained at room temperature was added phenol (20 g, 0.21 mol) by spatula. Following addition the mixture was allowed to stir overnight at room temperature after which time it was refluxed for 90 minutes. The excess phosphorus trichloride was distilled off at atmospheric pressure (b.pt. 75-77 ^{o}C). Distillation under reduced pressure afforded phenyl phosphorodichloridite as a colourless liquid (16.2 g, 64%). B.pt. 75-85 ^{o}C 6-10 mm/Hg.

δ ^{31}P n.m.r. (ether) +177.0 p.p.m.

The Preparation of Methyl Phenyl Phosphorochloridite (136)

To a solution of methyl phosphorodichloridite (67, 8.8 g, 0.066 mol) and pyridine (5.2 g, 0.66 mol) in anhydrousether (~ 120 cm^3) at -78 ^{o}C (CO_2/acetone bath) was added dropwise with stirring a solution of phenol (6.2 g, 0.066 mol) in the same solvent (50 cm^3), this solution at room temperature. Upon completion of addition, 90 minutes, the cooling bath was removed and the solution allowed to warm to room temperature and was stirred at this temperature for a further 30 minutes. Pyridinium

hydrochloride was removed by filtration (celite-aided), the filtrate being well washed with ether, and the solvent removed under reduced pressure to leave a yellow oil (12.1 g). Distillation under reduced pressure gave methyl phenyl phosphorochloridite as a colourless liquid (10.5 g, 83%). B.pt. 66-72 ^{o}C 0.65 mm/Hg.

δ ^{31}P n.m.r. (ether)	+160.1 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 3.75(d, 3H, J_{P-H} = 12Hz);
	6.9-7.4(m, 5H, aromatics).

The Preparation of 5'-O-Tritylthymidine-3'-O-(isobutyl phenyl phosphate)(139)

(a) Prior to the attempted preparation of (139) using the Arbusov reaction, it was initially prepared by the method of Letsinger.[62]

To a solution of phenyl phosphorodichloridite (0.29 g, 0.0015 mol) and pyridine (0.12 g, 0.0015 mol) in methylene chloride (2 cm^3) at -78 ^{o}C was added dropwise by syringe (over a 3 minute period) a solution of 5'-O-tritylthymidine (0.73 g, 0.0015 mol) in the same solvent (2 cm^3). To this solution, still at -78 ^{o}C, was added, by syringe, a solution of isobutanol (0.11 g, 0.0015 mol) and pyridine (0.12 g, 0.0015 mol) in the same solvent (2 cm^3). The cooling bath was removed and the solution allowed to warm to -10 ^{o}C at which point a solution of iodine (0.38 g, 0.0015 mol) in THF/water, 2:1 (v/v) was added. The ^{31}P n.m.r. spectrum contained three major absorptions at -6.1 p.p.m., -7.1 p.p.m. and -8.1 p.p.m. present as 34%, 28% and 38% of the mixture respectively. The reaction mixture was poured into a 250 cm^3 separating funnel, the reaction flask being washed out well with methylene chloride. The organic phase was then washed with, (i) sodium thiosulphate solution (100 cm^3), (ii) sodium bicarbonate solution (100 cm^3) and then with water (2 x 100 cm^3) before being dried over Na_2So_4. Following filtration the solvent was removed under reduced

pressure to leave a gum which was taken up in methylene chloride (1 cm^3) and precipitated into cold hexane to give a white powder (0.6 g). The ^{31}P n.m.r. spectrum of the powder contained two absorptions at -7.1 p.p.m. and -8.1 p.p.m. The crude material was purified by column chromatography (silica gel using chloroform : ethanol 97:3v/vas eluting solvent). Pure fractions (R_f = 0.28) were pooled, dissolved in chloroform and precipitated into cold hexane to give a white powder which was filtered and dried (0.2 g, 20%). This was shown by n.m.r. to be the required phosphate.

δ ^{31}P n.m.r. (CH_2Cl_2)	-7.1 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 0.75-0.95 (2 x d, 6H, J = 8Hz);
	1.31 (s, 3H); 2.2-2.65 (m, 2H);
	3.18-3.48 (m, 2H); 3.6-3.9 (m, 2H);
	4.05-4.35 (m, 1H); 5.01-5.35 [m(b), 1H];
	5.01-5.34 [m(b), 1H]; 6.15-6.45 [t(b), 1H];
	7.0-7.6 [m(b), 21H]; 9.65-9.85 [s(b), 1H].

In similar subsequent experiments the species having chemical shifts of -6.1 p.p.m. and -8.1 p.p.m. in the ^{31}P n.m.r. spectrum were shown to be di-isobutyl phenyl phosphate and di-5'-O-tritylthymidine-3'-O-(phenyl phosphate) respectively, arising in this experiment due to disubstitution of the starting material.

(b) To a solution of methyl phenyl phosphorochloridite (0.25 g, 0.0013 mol) in methylene chloride (2 cm^3) at -78 ^oC (CO_2/acetone bath) was added a solution of 5'-O-tritylthymidine (0.64 g, 0.0013 mol) and diisopropylethylamine (0.67 g, 0.0052 mol) in the same solvent (3 cm^3). Following this, one molar equivalent of bromine (0.2 g, 0.0013 mol) was added as a solution in methylene chloride. After ^{31}P n.m.r. analysis of the resulting phosphorobromidate a solution of isobutanol (0.096 g,

0.0013 mol) and 1-methylimidazole (0.43 g, 0.0052 mol) in methylene chloride (2 cm^3) was added. The ^{31}P n.m.r. spectrum recorded within five minutes showed a major absoprtion at -7.1 p.p.m. Following work-up as before and purification by column chromatography (silica gel using chlorofrom :ethanol 97 : 3 as eluting solvent) the species at -7.1 p.p.m. was isolated in 40% yield and was shown by n.m.r. spectroscopy to be identical to the sample of (139) prepared by the standard route.

The Preparation of 5'-O-Tritylthymidine-3'-O-(3'-O-acetylthymidyl phenyl phosphate) (143).

The fully protected dimer was prepared in an identical manner to the previous phosphate (139) but using 3'-O-acetylthymidine (11a) in place of the isobutanol and was isolated in 24% yield after column chromatography and preparative thin layer chromatography.

δ ^{31}P n.m.r. (CH_2Cl_2)	-6.9 p.p.m., -7.3 p.p.m.
^1H n.m.r. ($CDCl_3$)	δ 1.4-1.35 (2 x s, 6H); 1.8-1.9 (s, 3H); 1.95-2.4 (m, 4H); 3.05-3.33 (m, 2H); 3.75-4.0(m, 2H); 4.01-4.4 (m, 3H); 4.88-5.15 (m, 1H); 6.05-6.35 [t(b), 2H]; 6.75-7.35 (m, 22H); 9.4-9.65 [s(b), 2H].

Attempted Preparation of o-Chlorophenyl Methyl Phosphorochloridite (141)

To a solution of methyl phosphorodichloridite (4.0 g, 0.03 mol) and pyridine (2.3 g, 0.03 mol) in anhydrous ether (200 cm^3) at -82 ^oC (CO_2/ether bath) was added dropwise with stirring a solution of o-chlorophenol (3.8 g, 0.03 mol) in the same solvent (150 cm^3). Upon completion of addition (2½ hours) the cooling bath was removed and the

solution allowed to warm to room temperature. Following ^{31}P n.m.r. analysis (Figure 5.5 (ii), page 100) the pyridinium hydrochloride was removed by filtration (celite aided) under nitrogen, and the solvent removed under reduced pressure to leave a yellow oil (5g, 75% crude recovery). Attempted distillation under reduced pressure succeeded only in isolating ~ 0.5 g of di-o-chlorophenyl methyl phosphite.(142) B.pt. 120-125 ^{o}C 0.2 mm/Hg.

The Preparation of Diethyl Phosphorochloridite

This compound was prepared using the method of Saunders et al.[145] A solution of phosphorus trichloride (25.1 g, 0.18 mol) and triethyl phosphite (60.7 g, 0.37 mol) was gently refluxed for 30 minutes. Distillation at atmospheric pressure afforded diethyl phosphorochloridite as a colourless liquid (31 g, 37%). B.pt. 145-148 ^{o}C. (lit[146] 53-54 ^{o}C 25 mm/Hg).

δ ^{31}P n.m.r. (ether) +165.6 p.p.m.

The Preparation of Diethyl Phenyl Phosphite (144b)

To a solution of phenyl phosphorodichloridite (5.3 g, 0.027 mol) and pyridine (4.3 g, 0.054 mol) in ether (10 cm^3) at room temperature was added dropwise with stirring a solution of ethanol (2.5 g, 0.054 mol) in the same solvent (8 cm^3). Pyridinium hydrochloride was removed by filtration (celite-aided), the filtrate being well washed with ether, and the solvent removed under reduced pressure to leave a colourless oil (4.9 g). Distillation under reduced pressure afforded diethyl phenyl phosphite (1.8 g, 31%). B.pt. 94-98 ^{o}C 3.5 mm/Hg.

δ ^{31}P n.m.r. (ether) +132.9 p.p.m.

^{1}H n.m.r. ($CDCl_3$)	δ 1.2-1.4 (t, 6H, J = 6Hz);
	3.82-4.18 (dq, 4H, $J_{P-H} \sim J_{H-H}$ = 6Hz);
	6.88-7.33 (m, 5H, aromatics).

The Preparation of o-Chlorophenyl Diethyl Phosphite (144d)

To a solution of diethyl phosphorochloridite (3.8 g, 0.024 mol) in ether (40 cm^3) at 0 °C (ice-bath) was added dropwise with stirring a solution of o-chlorophenol (3.3 g, 0.026 mol) and pyridine (2g, 0.026 mol) in the same solvent (35 cm^3). The addition was carried out over a period of 10 minutes after which time the cooling bath was removed and the solution allowed to warm to room temperature. Pyridinium hydrochloride was removed by filtration through a column of activated alumina (1 x 10 cm) and the solvent removed under reduced pressure to leave a yellow oil (6.3 g). Distillation under reduced pressure afforded o-chlorophenyl diethyl phosphite as a colourless oil (3.6 g, 60%). B.pt. 96-98 °C 0.25 mm/Hg.

δ ^{31}P n.m.r. (ether)	+133.1 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 1.24-1.45 (t, 6H, J = 6Hz);
	3.88-4.2 (dq, 4H, $J_{P-H} \sim J_{H-H}$
	= 6Hz); 6.84-7.38 (m, 4H).

Characteristics of the Aryl Diethyl Phosphites (144)

Ar.	B.pt	$\delta^{31}P$ n.m.r. (ether)	Yield
para MeO Ph (144a)	-	+134.7 p.p.m.	25%*
Ph (144b)	94-98 °C 3.5mm/Hg	+132.9 p.p.m.	31%
para Cl Ph (144c)	92-96 °C 0.28mm/Hg	+133.5 p.p.m.	25%
ortho Cl Ph (144d)	96-98 °C 0.25mm/Hg	+133.1 p.p.m.	60%
3,5- DiCl Ph (144e)	114-118 °C 0.2mm/Hg	+134.1 p.p.m.	38%
2,4- DiCl Ph (144f)	120-125 °C 7mm/Hg	+134.1 p.p.m.	55%
2,6- DiCl Ph (144g)	117-120 °C 0.2mm/Hg	+135.7 p.p.m.	31%
para NO_2 (144h)	-	+132.9 p.p.m.	90%*

*These are crude recoveries as both phosphites were found to decompose upon attempted distillation.

The Phosphites (144a-144h) compiled in the table were all prepared in the way as described for o-chlorophenyl diethyl phosphite (144d) with the exception of (144b) which was prepared from phenyl phosphorodichloridite (140) and ethanol as described.

The Reaction of Diethyl p-Nitrophenyl Phosphite (144h) with Isobutanol in the Presence of Tetrazole (72)

To a solution of diethyl p-nitrophenyl phosphite (0.6 g, 0.0023 mol) and isobutanol (0.17 g, 0.0023 mol) in acetonitrile (10 cm^3) at room temperature was added dropwise with stirring a solution of tetrazole (0.65 g, 0.0093 mol) in the same solvent (8 cm^3). The consumption of the starting material was monitored by ^{31}P n.m.r. spectroscopy. After 25 minutes the starting material [δ ^{31}P n.m.r. (CH_3CN) +134.3] had been replaced by an absorption at +138.8 p.p.m. At this point sulphur

(0.11 g, 0.0035 mol) was added and the mixture stirred for 30 minutes. The reaction mixture was filtered, the filtrate being well washed with methylene chloride. The organic phase was washed with 2N sodium hydroxide (2 x 50 cm^3) and then with water before being dried (Na_2SO_4). Following filtration, the solvent was removed under reduced pressure to leave a pale yellow oil (0.23 g). The ^{31}P n.m.r. spectrum of the crude material showed one absorption at +67.3 p.p.m. When this spectrum was re-recorded on a 1KHz sweep width the peak at +67.3 p.p.m. was shown to contain several phosphorus species, presumably due to a statistical mixture of all possible thiophosphates. Attempted distillation under reduced pressure did not result in purification of the major component. Although there is a mixture of compounds present, from the stoichiometries of reagents used the 'H n.m.r. still contained absorptions due to the isobutyl group and the ethyl groups in the ratio of 1:2.

'H n.m.r. ($CDCl_3$)	δ 0.82-0.96 (d, 6H, J = 7Hz)
	1.18-1.31 (t, 6H, J = 6Hz);
	1.66-2.1 (m, 1H); 3.61-3.78(m, 2H);
	3.82-4.24 (m, 4H, dq not really
	resolvable).

N.B. The reactions of all the aryl diethyl phosphites prepared with isobutanol in the presence of tetrazole were conducted in the same way as described above for diethyl p-nitrophenyl phosphite.

The Preparation of 2,6-Dichlorophenyl Methyl N,N-dimethylphosphoramidite (146)

To a solution of chloro-N,N-dimethylaminomethoxyphosphine (0.2 g, 0.065 mol) in ether (150 cm^3) at -78 ^oC (CO_2/acetone bath) was added dropwise with stirring a solution of 2,6-dichlorophenol (10.6 g, 0.065 mol) and triethylamine (26.3 g, 0.26 mol) in the same solvent (100 cm^3). Upon

completion of addition (30 minutes) the cooling bath was removed and the solution allowed to attain room temperature and was stirred at this temperature for 30 minutes. Triethylammonium hydrochloride was removed by filtration (celite-aided) and the solvent removed under reduced pressure to leave a yellow oil (12.4 g, 71% crude recovery). This compound proved very difficult to distil, only a small amount of distillate being collected (2.1 g, 16%), even under high vacuum. B.pt. 122-125 ^{o}C. 0.0001mm/Hg.

δ ^{31}P n.m.r. (ether)	+150.0 p.p.m.
^{1}H n.m.r. ($CDCl_3$)	δ 2.67 (d, 6H, J_{P-H} = 10Hz);
	3.6 (d, 3H, J_{P-H} = 12Hz);
	6.73-7.3 (m, 3H, aromatics)
mass spec m/e M^{+}	269, 161, 106 (100%), 63, 29.

The Preparation of 5'-O-Dimethoxytritylthymidine-3'-O-(isobutyl methyl phosphate) (112a) using the Exchange Reaction.

To a solution of (146, 0.11g, 0.0004 mol) in anhydrous acetonitrile (1 cm^3) at room temperature was added dropwise with stirring a solution of 5'-O-dimethoxytritylthymidine (0.22 g, 0.0004 mol) and tetrazole (0.031 g, 0.0004 mol) in the same solvent (1 cm^3). Following ^{31}P n.m.r. spectroscopic analysis a solution of isobutanol (0.03 g, 0.0004 mol) and tetrazole (0.12 g, 0.0017 mol) in acetonitrile (2 cm^3) was added. After 90 minutes the absorption due to the starting phosphite (+137.1 p.p.m. and +136.1 p.p.m.) was no longer present and the mixture was treated with a solution of iodine (0.18 g, 0.0006 mol) and pyridine (0.049 g, 0.0006 mol) in THF/H_2O (3 cm^3, 2:1 v/v). The reaction mixture was extracted with methylene chloride, the organic phase being washed with (i) sodium thiosulphate solution, (ii) sodium bicarbonate solution and (iii) water (2 x 100 cm^3) before being dried over $MgSO_4$. Following

filtration, the solvent was removed under reduced pressure to leave a yellow foam which was dissolved in methylene chloride (2 cm^3) and precipitated into cold hexane (150 cm^3) to give a white powder which was filtrered and dried (0.15 g, 61% crude recovery). Purifcation was effected by preparative thin layer chromatography on silica gel using chloroform : ethanol : triethylamine, (97.2:1v/v)as eluting solvent. Two bands were scraped off, extracted with methanol : ether (30 : 70 v/v). The higher R_f material (0.27) was due to the required phosphate (112a) (0.051 g, 20%), it being identical on tlc and spectroscopically to the same material prepared in the Arbusov reaction route.

The lower R_f material (0.19) was shown to be due to di-5'-O-dimethoxytritylthymidine-3'-O-(methyl phosphate) (149).

δ ^{31}P n.m.r. (CH_2Cl_2) -2.0 p.p.m.

From the ^1H n.m.r. (300 MHz, $CDCl_3$) it was difficult to assign all the protons in the molecule due to the excessive amount of aromatic protons present due to two dimethoxytrityl protecting groups (26 protons).

Major characteristics. δ 1.2 (s, 6H); 3.68 (d, 3H, J_{P-H} = 12Hz); 3.77 (s, 12H); 6.7-7.4 (m, 26H, including AA'XX' pattern).

Reagents and Solvents for Solid Phase Synthesis

All solvents were purified under an inert atmosphere of dry nitrogen. Acetonitrile was refluxed over and distilled from calcium hydride, as was methylene chloride. Pyridine was refluxed over and distilled from calcium hydride twice and refluxed and distilled from tosyl chloride once. DMF was distilled under reduced pressure.

Dioxan was passed through a column of basic alumina and then refluxed over and distilled from sodium benzophenone ketal immediately prior to use. Initially, all solvents (with the exception of dioxan) were stored over activated 3A molecular sieves and under nitrogen in rubber septum cap sealed bottles, the solvents being transferred with dry syringes. Subsequently, following the advice of Gait,[54] the solvents were re-purified and not stored over molecular sieves.

Trichloroacetic acid was dried in vacuo at 50 °C over phosphorus pentoxide overnight and recrystallised from chloroform. 1-methylimdazole was distilled under reduced pressure. Diisopropylethylamine was distilled from calcium hydride at atmospheric pressure. Triethylamine was distilled from potassium hydroxide pellets at atmospheric pressure. Thiophenol, acetic anhydride and perchloric acid were used without purification.

The major solid phase experiment described is that which led to a 34% yield of thymidylyl (3'→5')-thymidine. The other oligomer preparations attempted were simple extended cycles of virtually the same protocol with variarion of solvent, concentration and ultimately reagents.

Solid Phase Synthesis of Thymidylyl (3'→5')-Thymidine, TpT (20)

The solid phase experiment consisted of several stages.

1. Phosphite preparation
2. Terminal deprotection on the support and trityl analysis.
3. The Arbusov reaction.
4. Activation of the phosphorobromidate and phosphorylation.
5. Deprotections, including cleavage from the support.
6. HPLC analysis.

1. Phosphite Preparation

This was carried out the day prior to each solid phase experiment conducted.

To a solution of dimethyl phosphorochloridite (0.12 g, 0.00099 mol) in methylene chloride (1 cm^3) at room temperature was added dropwise by teflon cannula needle a solution of 5'-O-dimethoxytritylthymidine (0.54 g, 0.00099 mol) and diisopropylethylamine (0.13 g, 0.00099 mol) in the same solvent (2 cm^3). ^{31}P n.m.r. analysis showed a major absorption at +140.4 p.p.m. due to the required phosphite and a minor absorption at +10.7 p.p.m. due to dimethyl phosphite. The solution was made up to 5 cm^3 (~ 0.2M solution) with methylene chloride and stored in a storage vial under nitrogen in the deep freeze until required.

2. Terminal Deprotection and Trityl Analysis

50 mg of LCAA/CPG was weighed out and packed into the Omnifit column. The loading of the first nucleoside residue on the support was 0.00004 mol/g. Therefore, 50 mg of the support contained, attached through its 3'-position, 0.0000022 mol of the first nucleoside residue.

Reagents and solvents were passed through the apparatus using a pressure of dry nitrogen at a flow rate of ~ 2 cm^3/minute.

Prior to deprotection the support was washed with

1. Pyridine (2 cm^3)
2. Acetonitrile (2 cm^3)
3. DMF (4 cm^3)
4. Pyridine (4 cm^3)
5. CH_2Cl_2 (3 cm^3)

in order to wash clear of the support any polar impurities that may be present.

The support was then treated with 3 cm^3 of a 10% solution of trichloroacetic acid (TCA) in methylene chloride. Upon immediate contact with the support the solution colour immediately turned bright orange, indicative of the dimethoxytrityl cation. Following the acid treatment TCA residues were washed from the support with acetonitrile (8 cm^3). The TCA washes were combined and the solvent removed under reduced pressure. The orange oil obtained was diluted to 250 cm^3 with 60% perchloric acid : ethanol (6 : 4 v/v) and the absorbance measured at 495 nm. From this it was calculated that 0.0000021 moles of the dimethoxytrityl cation had been released from the support, thereby indicating a deprotection yield of 95%.

3. The Arbusov Reaction

2 cm^3 (0.000396 mol) of the stock solution of the dimethyl nucleoside phosphite (107a) was syringed into Flask B (See Figure 6.2, page 110) of the apparatus. This amount corresponds to a 188% excess of phosphite over the 5'-hydroxyl attached to the support. The solution was cooled to 0 ^{o}C (ice bath) and one molar equivalent of bromine was added as a solution in methylene chloride. The ^{31}P n.m.r. spectrum of the reaction mixture showed two absorptions at +4.8 p.p.m. and +4.4 p.p.m. characteristic of the phosphorobromidate (110a).

4. Activation and Phosphorylation

1-Methylimidazole [0.065 g, 0.00079 mol, 2 molar equivalents w.r.t. (110a)] and diisopropylethylamine (0.051 g, 0.00039 mol) were injected into Flask C (see Figure 6.2, page 110) of the apparatus. The phosphorobromidate (110a) was transported to Flask C through a teflon cannula and the contents of Flask C were then slowly (the

pressure of positive nitrogen having been reduced) passed over the CPG support over a period of 45 minutes with agitation. Following the phosphorylation process, residues were washed from the support with DMF (8 cm^3), acetonitrile (4 cm^3) and methylene chloride (4 cm^3) after which the support was "dried" by blowing a positive pressure of dry nitrogen through the omnifit column for 5 minutes.

5. Deprotections

The solid phase apparatus was dismantled and the CPG support was treated, in a 10 cm^3 round bottomed flask, with a mixture of thiophenol (154), triethylamine and dioxan (1:1:2 v/v) for 45 minutes with agitation. This procedure removes the internucleotide methyl esters. The support was filtered at the pump, and washed with methanol (4 x 10 cm^3) and diethyl ether (2 x 10 cm^3) and then air dried.

The support was transferred to a 25 cm^3 round bottomed flask and 10 cm^3 of approximately 35% ammonia solution was added. The flask was sealed with a rubber septum cap which was tightly attached with copper wire. The flask was heated at 50 ^{o} overnight. This procedure cleaved the dimer from the support.

The ammonia solution was removed by Pasteur pipette) and transferred to a 100 cm^3 round bottomed flask. The CPG was washed with water (2 x 10 cm^3) and these washings combined with the ammonia. The solvent was removed under reduced pressure and the residue coevaporated with water (2 x 20 cm^3). The resulting material was taken up in 80% acetic acid (15 cm^3) and stirred for 30 minutes at room temperature. This removes the terminal dimethoxytrityl protecting group.

The solvent was removed under reduced pressure and the residue coevaporated with toluene (2 x 20 cm^3). The resulting material was

dissolved in water (5 cm^3) and extracted with ether (6 x 10 cm^3, vigorous shaking). The ether (top layer) was removed with a Pasteur pipette after each washing. The aqueous layer was filtered through a cottonwool plug and the water removed under reduced pressure. The residue was taken up in 2 cm^3 of water prior to hplc.

6. HPLC Analysis

Anion-exchange high pressure liquid chromatography was used to analyse the crude product. The eluting conditions of Gait were employed[129] which use a column temperature of 25 ^oC and buffers of 1 mM and 300 mM KH_2PO_4 pH 6.3, the gradient running from 100% 1mM, 0% 300 mM to 0% 1mM, 100% 300mM over 60 minutes. The u.v. detector was set at 265 nm. Authentic thymidine (T) and thymidylyl (3'→5')-thymidine (TpT), obtained from the Aldrich Chemical Company, were used as standards. Authentic T and TpT were, under these conditions, found to elute with retention time of 1.7 minutes and 2.2 minutes respectively.

The crude reaction mixture, when subjected to these conditions, contained two major peaks in the chromatogram with retention times of 1.75 minutes and 2.1 minutes, and were present as 31% and 69% of the mixture respectively (by electronically calculated peak areas).

As two chromophores are present in TpT and one in T, then the real contribution of TpT to the mixture was 34%.

Initial experiments were carried out with the aim of synthesising a hexamer containing just thymidine. This required five phosphorylations and five deprotections. The phosphorylations were carried out using approximately 30 molar equivalents over the polymer bound nucleoside per phosphorylation. At his stage quantitative trityl analysis was not carried out but just viewed visually, the deprotections seeming reasonably consistent. Subsequent solid phase experiments were

conducted with various modifications. The trityl deprotective analysis was carried out quantitatively as described with 60% perchloric acid : ethanol (6 : 4 v/v) and this was very indeterminate indicating insufficient deprotection at one step and execessive deprotection at another midway through an experiment.

Polar washing steps were introduced, acetonitrile and DMF, in the belief that the methylene chloride washes previously used were not of sufficient polarity to wash clear of the support TCA reaction residues.

A capping step was introduced, which was a mixture of DMAP (2.5 g, 0.2 mol) and acetic anhydride (0.23 g, 0.0023 mol) in pyridine (30 cm^3). 3 cm^3 of this solution was passed over the support when required following each phosphorylation.

The phosphorylation reaction times were varied between 20 and 50 minutes with varying excesses of reagent being used, from 30 molar equivalents to 188 molar equivalents per phosphorylation.

The solvent that the phosphorylation was carried out in was varied, initially to acetonitrile [the ^{31}P n.m.r. chemical shift of the phosphorobromidate (110a) in this solvent coming at +3.8 p.p.m. and +3.6 p.p.m.] with no significant increase in the degree of phosphorylation. The phosphorobromidate, in a subsequent experiment, was generated in pyridine as solvent, the ^{31}P n.m.r. chemical shift in this solvent being -5.7 p.p.m. (the diastereoisomrs not being resolved). This is a dramatic change in the chemical shift from when the phosphorobromidate was generated in acetonitrile and methylene chloride as solvent, and, with hindsight, may be due to the demethylated species analogous to (158), Figure 6.7 page 119) as reported by Thuong and Asseline.[123]

In no solid phase experiment, where methyl was present as the internucleostide protecting group, was a 34% coupling efficiency bettered.

The Use of o-Chlorophenyl as Internucleotide Protecting Group.

Preparation of Bis-(diisopropylamino)methoxyphosphine (76)

This was prepared by the method of Caruthers.[68] To a solution of methyl phosphorodichloridite (9.4 g, 0.071 mol) in anhydrous diethyl ether (150 cm^3) at -5 °C was added dropwise with stirring, a solution of diisopropylamine (45 g, 0.45 mol, 6 molar equivalents) in the same solvent (150 cm^3), this solution at room temperature. The addition took place over a period of 1 hour after which time the cooling bath was removed and the solution allowed to stir at room temperature overnight. Diisopropylammonium hydrochloride was removed by filtration (celite-aided) under nitrogen and the solvent removed under reduced pressure to leave a clear oil (12.5 g). Distillation under reduced pressure gave bis-(diisopropylamino)-methoxyphosphine as a clear oil (9.6 g, 52%), b.pt. 72-76 °C 0.15 mm/Hg.

δ ^{31}P n.m.r. (ether)	+131 p.p.m.
^1H n.m.r. $CDCl_3$	δ 1.5 (d, 24H, J = 7Hz); 3.24-3.75 (m, 4H, including prominent d, 3H, J_{P-H} = 12Hz).

Attempted Solid Phase Synthesis of TpT with o-Chlorophenyl as the Internucleotide Protecting Group.

The same solid phase apparatus as in previous experiments was

used, Figure 6.2, page 110.

To a solution of bis-(diisopropylamino)methoxyphosphine (0.24 g, 0.0009 mol) and diisopropylammonium tetrazolide (77, 0.18 g, 0.000 9 mol) in methylene chloride (2 cm^3), was added a solution of 5'-O-dimethoxytritylthymidine (0.5 g, 0.0009 mol) in the same solvent (2 cm^3). After 5 minutes, and following a ^{31}P n.m.r. analysis, a solution of o-chlorophenol (0.12 g, 0.0009 mol) and (77, 0.18 g, 0.0009 mol) in methylene chloride (1 cm^3) was added. The solution was cooled to 0 ^oC (ice bath) and one molar equivalent of bromine (0.16 g, 0.0009 mol) as a solution in the same solvent was added. Following solvent washes, as before, and terminal deprotection (10% TCA in methylene chloride, quantitative analysis as previously described indicated 100% deprotection) the solution of the phosphorobromidate (160) was mixed (in Flask C, Figure 6.2, page 110) with 1-methylimidazole (0.073 g, 0.0009 mol) and diisopropylethylamine (0.12 g, 0.0009 mol) and passed slowly over the 5'-hydroxyl attached to the CPG, over a period of 30 minutes with agitation at room temperature (the phosphorylating agent was present in about 400 times excess). Following the phosphorylation process, the support was washed with acetonitrile (4 cm^3), DMF (4 cm^3) and methylene chloride (4 cm^3), before being dried by blowing dry nitrogen through the omnifit column for 5 minutes.

In a 5 cm^3 round bottomed flask the CPG was treated with a solution of syn-2-nitrobenzaldoxime (0.07 g, 0.0004 mol) and N,N,N',N'-tetramethylguanidine (0.04 g, 0.0004 mol) in 1 cm^3 of dioxan: water (1 : 1 v/v) and the mixture agitated at room temperature overnight. Following this, the support was filtered off using a glass sinter and washed with dioxan : water (1 : 1 v/v) until the washings were no longer coloured. The solvent was removed under reduced pressure and the residue was treated with 80% acetic acid for 30 minutes at room temperature.

The solvent was removed under reduced pressure and the residue coevaporated with toluene (2 x 20 cm^3). The resulting material was dissolved in water (5 cm^3) and extracted with ether (6 x 10 cm^3). The aqueous layer was filtered through a cottonwool plug and the water removed under reduced pressure. The residue was taken up in 2 cm^3 of water prior to hplc analysis.

HPLC analysis was carried out on reversed phase using an isocratic gradient of 20 mM tetraethylammonium bicarbonate, pH 7.0, with 3% acetonitrile. Under these conditions, with a column temperature of 25 ^oC and a flow rate of 2 cm^3/minute, authentic thymidine eluted with a retention time of 1.77 minutes and authentic thymidylyl (3'$\longrightarrow$5')-thymidine eluted with a retention time of 5.9 minutes. The crude reaction mixture contained both of these peaks and the contribution to the mixture of thymidylyl (3'$\longrightarrow$5')-thymidine, TpT, was approxiamtely 30%, estimated from electronically calculated peak areas.

Time did not allow a more thorough invesitigation of the approach described above.

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