# Studies in Solid Supported Oligonucleotide Synthesis

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**Abstract**

Chemical synthesis of oligonucleotides by solid supported methods has been dominated by the use of triarylmethyl ether protecting groups at the 5'-hydroxyl site of the deoxyribose moiety. These groups, *eg.* 4,4'-dimethoxytrityl, are cleaved with acids. Here I describe studies in the application of the base labile 9-fluorenylmethoxycarbonyl group to the protection of the 5'-hydroxyl in deoxyoligonucleotide synthesis and in oligoribonucleotide synthesis.

Comercially available long chain alkylamine controlled pore glass resin was found to be unstable to the strong non-nucleophilic bases used to cleave the 9-fluorenylmethoxcarbonyl protecting group. After a model study to elucidate the mechanism of the cleavage reaction, a long chain alkylamine controlled pore glass resin, with a sarcosyl spacer group between the alkyl chain and the succinyl link to the first nucleoside, was synthesised. This sarcosyl resin was found to be stable to bases such as DBU and DBN.

Solid supported synthesis of chemically labelled oligonucleotides was also investigated. A monomer for oligonucleotide synthesis, which carried a 2,4-dinitrophenyl group for use as a fluorescent and immunogenic label, was designed and synthesised. This monomer was used to label deoxyoligonucleotides for use in hybridisation studies and polymerase chain reaction.

**Acknowledgements**

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**Contents**

## Abbreviations

| | |
|---|---|
| A | Adenosine |
| AUFS | Absorbance Units Full Scale |
| Bz | Benzoyl |
| C | Cytidine |
| dA | 2'-Deoxyadenosine |
| dC | 2'-Deoxycytidne |
| dG | 2'-Deoxyguanosine |
| DBN | 1,5-Diazabicyclo[4.3.0]non-5-ene |
| DBU | 1,8-Diazabicyclo[5.4.0]undec-7-ene |
| DMF | N,N-Dimethylformamide |
| DMTr | 4,4'-Dimethoxytrityl |
| DNP | 2,4-Dinitrophenyl |
| FMOC | 9-Fluorenylmethoxycarbonyl |
| G | Guanosine |
| HPLC | High performance liquid chromatography |
| MEM | Methoxyethoxymethyl |
| T | Thymidine |
| TCA | Trichloroacetic Acid |
| THF | Tetrahydrofuran |
| TIPDS | Tetraisopropyldisiloxane-1,3-diyl |
| tlc | Thin layer chromatography |
| TMS | Trimethylsilyl |
| U | Uridine |

# Introduction

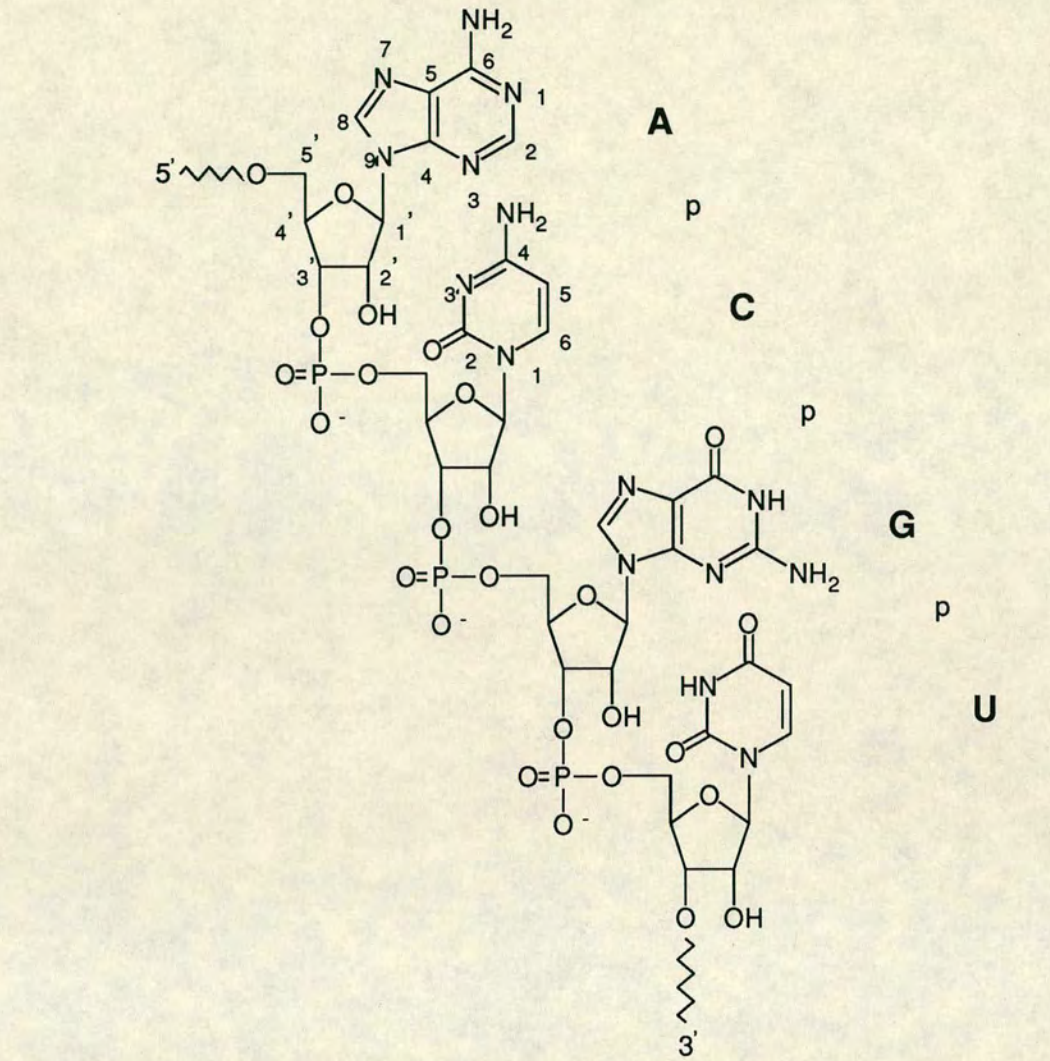
## 1.1 **Introduction**

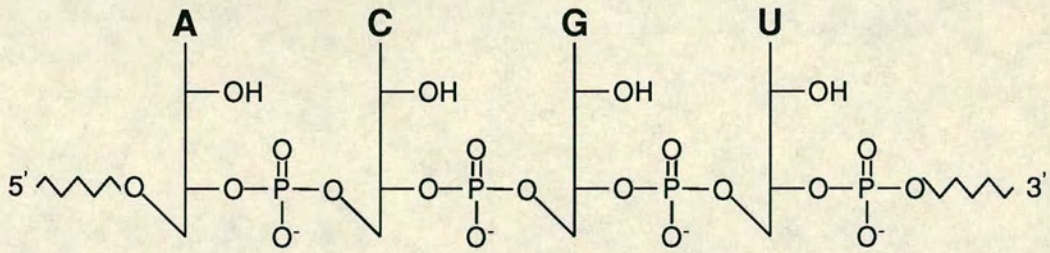
The primary chemical structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in single stranded form (Fig.1) consists of a chain of furanose rings linked by 3'-5' phosphodiesters, with each sugar carrying one of four possible heterocyclic bases in a beta configuration at the 1'-sugar site. For DNA the sugar ring is D-deoxyribose and the bases are adenine, guanine, cytosine and thymine. In the case of ribonucleic acid the sugar ring is D-ribose and the 4 bases adenine guanine, cytosine and uracil (Fig.2).

The sequence of bases along the the chain is vital to successful protein synthesis. DNA in the cell nucleus acts as a template in the synthesis of messenger-RNA(m-RNA) of complementary sequence and this sequence in its mature form codes for amino-acids at the ribosome where proteins are synthesised. The sequence of the RNA is read as a triplet code, each amino-acid having a different 3-base code to describe it. Transfer-RNA (t-RNA) transfers the code between RNA and proteins. Each t-RNA carries an amino-acid and a recognition site with the appropriate triplet code for that amino-acid. By hybridising to the m-RNA at the ribosome the correct amino-acid is delivered to the correct place in the growing protein.

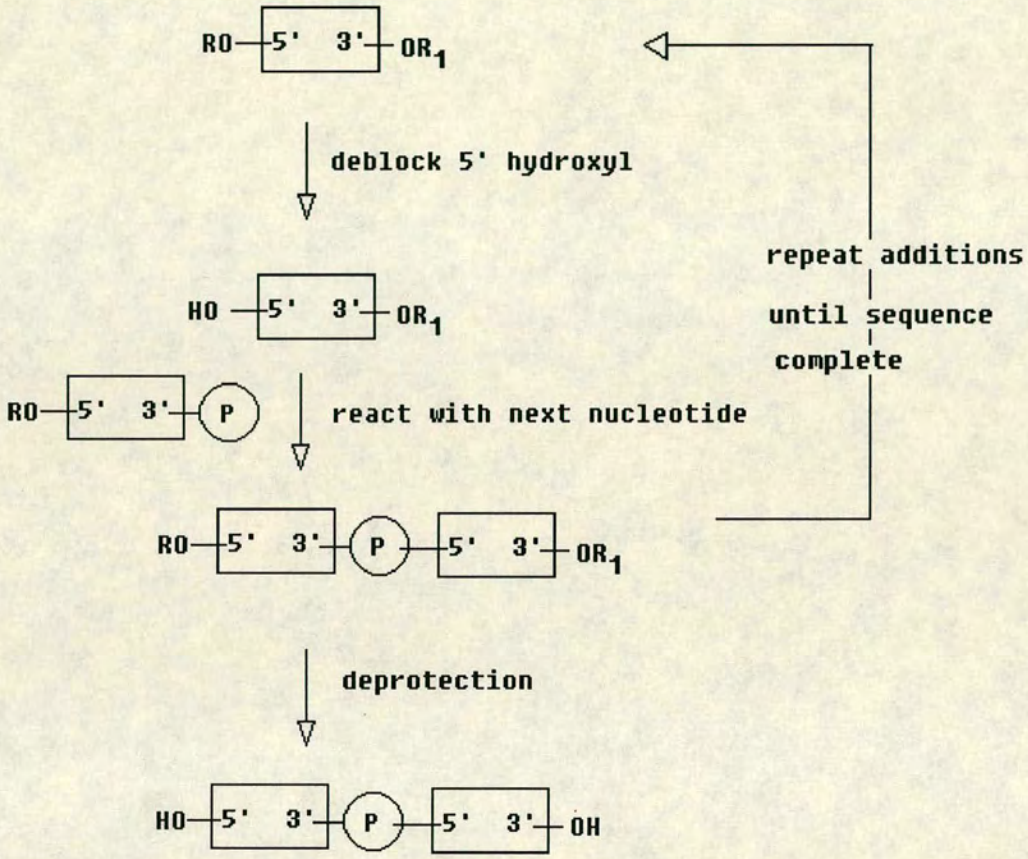
Chemical synthesis allows oligonucleotides of specific sequence to be prepared for a wide variety of applications. For molecular biology hybridisation probes, primers and entire genes can be prepared. Recent discoveries in structure and properties of RNA[1] have led to much interest in the synthesis of oligoribonucleotides. In chemical synthesis[2], nucleotide monomers are successively added to an oligonucleotide chain until the sequence is complete. The formation of the internucleotide link must be directed exclusively between the 3' and 5'-hydroxyl functions and this is achieved by protection of all other nucleophilic moieties. Thus in a synthesis, working in the normal way from 3' to 5' end of the oligonucleotide, the initial monomer would be a 3'-protected nucleoside which could the be coupled with a 5'-protected nucleotide (Scheme 1). For addition of the next nucleotide, the 5'-protecting group must be cleaved off. When the sequence is complete permanent protecting groups on exocyclic amine functions can be cleaved along with the 3'-protecting group and any phosphorus protecting groups, to leave the free oligonucleotide.

The concept of solid supported synthesis of polymers was introduced by Merri field[3] and initially

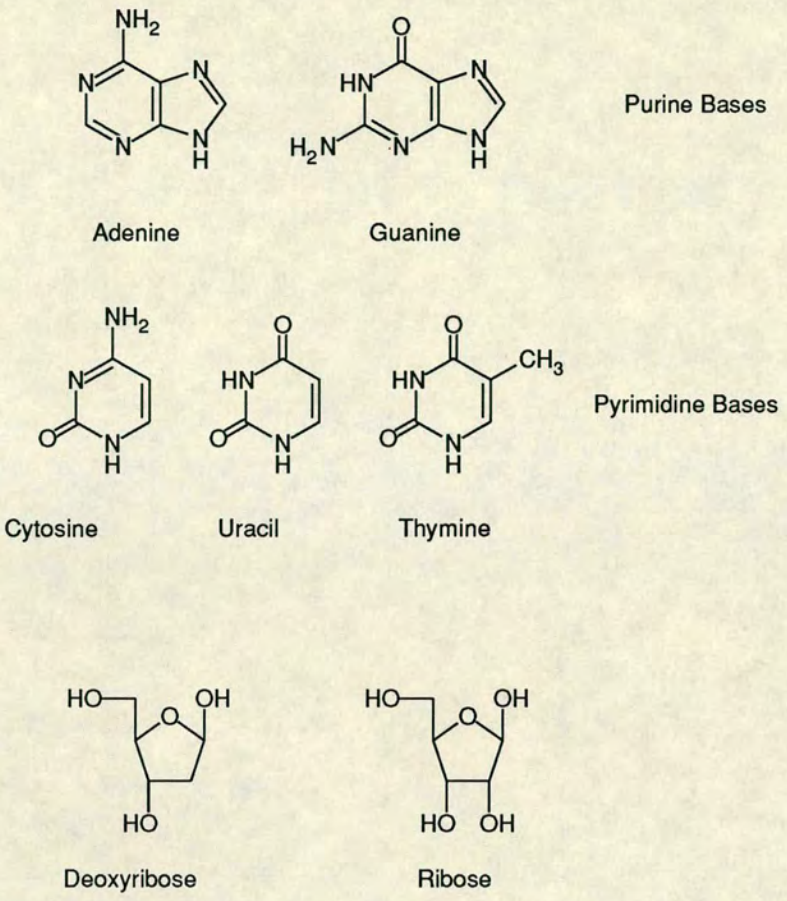



( Figure 1. Oligonucleotide Primary Structure)




( Scheme 1. Oligonucleotide Synthesis)




( Figure 2. Bases and sugars in Oligonucleotides)

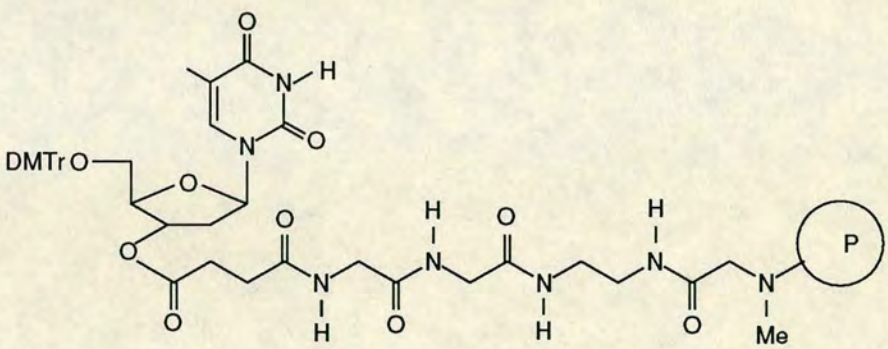
applied to peptide synthesis. In solid supported oligonucleotide synthesis the protecting group on the terminal 3'-nucleoside is replaced by a linker to a solid insoluble particle. Each reaction performed on the oligonucleotide is then pushed to completion with excess reagents which can then be washed away and the synthesis completed without the need for intermediate purification steps. Solid phase synthesis is therefore much quicker than solution synthesis for small scale work. A 20mer can easily be synthesised by solid phase methods in a single day whereas a synthesis in solution would require months to complete. Because the procedure amounts to a series of soaking and washing steps at room temperature, solid phase synthesis has been successfully automated.

## 1.2 **The Solid Support**

Solid phase synthesis, first introduced by Merrifield for peptides[3], was initially applied to oligonucleotide synthesis in 1965 using low crosslinking "popcorn" polymer and phosphotriester chemistry to produce the dimer d(CpG).[4]

Early attempts at solid phase oligonucleotide synthesis involved organic polymers as solid supports. However these compounds swell to varying degrees in different solvents, interact with solvents and in most cases are strongly hydrophobic. In addition they tend to adsorb the growing oligonucleotide chain onto their surface making it less accessible to synthetic procedures[2].

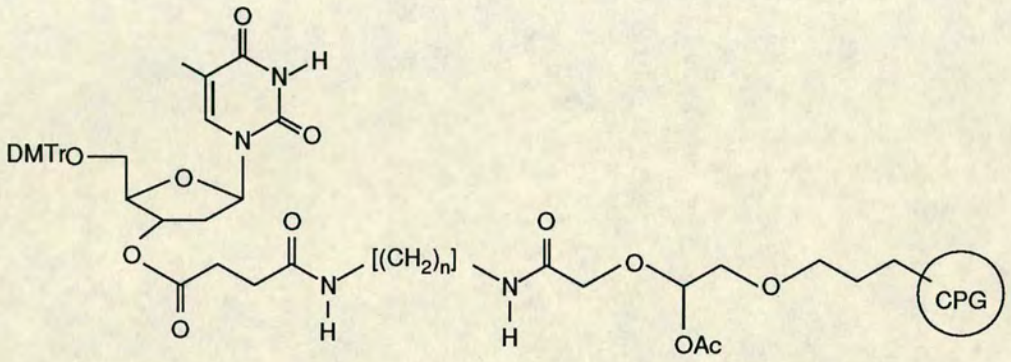
More inert matrices were developed in the shape of polydimethylacrylamide-kieselgel, a kieselgel framework with polydimethylacrylamide trapped in its pores. This support, used in phosphotriester synthesis, is capable of high loading ( up to 200micromol/g) and has good wetting properties ensuring the growing oligonucleotide contacts all synthesis reagents. However it is still prone to swelling (Fig. 3).




( Figure 3.)

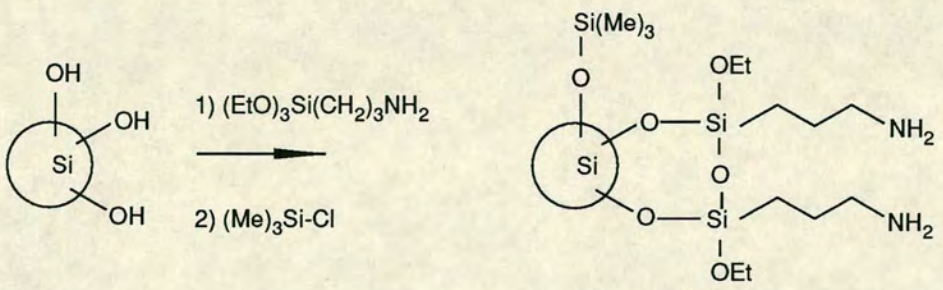
Completely rigid inert inorganic supports have also been developed and are now commonly used. The nonswellable nature of these matrices allow solvents and reagents to be pumped through during automated synthesis. Alkyl functionalised silica gel such as fractosil[5], the first of these substances, has a tendency to be easily fragmented releasing fine particles to the detriment of most automated synthesisers.

The support of choice is now long chain alkylamine controlled pore glass[6] (Fig. 4). This material is prepared commercially from borosilcate glass, the borates being etched out by acid to leave pores of regular size. Resins may have pore size of 500 or 1000 angstroms in diameter, with 500 angstrom being used in most commercial supports. Recently it has been noted[7] that supports with larger pore size give more homogeneous results for long oligonucleotides although at high pore size(eg >1000 angstroms) resins do become more fragile.



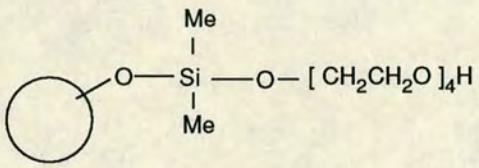

( Figure 4.)

Silica supports are functionalised by reaction of surface silanols with 3-aminopropyl-trialkoxysilane and unreacted hydroxyls are capped with trimethylsilyl chloride (Scheme 2). The amine groups can then be reacted with suitable groups to extend the chain and form the desired linker. Recent work suggests that the nature of this linkage can significantly affect the purity of oligonucleotide, especially if the sequence is long (ie.>50). It has been shown that the quality of synthesis is improved by increasing length and rigidity of the spacer[7].

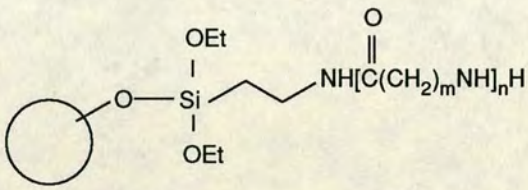



( Scheme 2. Functionalisation of silica supports)

In the same study it was noted a polyethylene glycol spacer (Fig. 5 ) performed well and an efficiency of greater than 99% was achieved in oligonucleotide synthesis with this linker. This is explained by dipole/dipole interactions which are thought to cause this linkage to remain in an extended form. Conversely a polyglycine spacer (Fig. 6, m=1 ) would be expected to adopt some form of folded structure due to hydrogen bonding between adjacent amide moieties, and this type of linkage did give a very poor quality product, which was a mixture of oligonucleotides, and a low coupling efficiency of 92%. When amide bonds were moved apart, by increasing alkyl chain length between them, the product purity was improved (increase in n in Fig 6).




( Figure 5.)




( Figure 6.)

Controlled pore glass can be obtained commercially already functionalised with long chain alkylamines, where the alkyl chain is 14 carbon units in length. This support has the advantages of being both rigid and robust, allowing high flow rates and solvent pressures to be used. The resin has good wetting properties, functional groups are normally accessible in all solvents and the matrix is chemically inert to all but very strong alkalis. Problems with surface adsorbed oligonucleotides do not occur and small adsorbed molecules can usually be washed off easily. Because of the uniform pore size, diffusion is the same through all particles and functionalisation is easy.

Attachment of the first nucleoside to the amine group of the long chain alkylamine spacer on the resin is normally by means of a further succinyl linker. The 5'-protected nucleoside is succinylated at the 3'-hydroxyl position with succinic anhydride in dry pyridine. Coupling of the 3'-succinate with the resin can be either by direct N,N-dicyclohexylcarbodi-imide(DCC) activated condensation or by an active ester such as 4-nitrophenol, which can be prepared by a DCC coupling reaction. This latter approach lessens the risk of precipitates of N,N-dicyclohexylurea(DCU) becoming mixed with the resin and the ester is stable during the long coupling time that is required. Alternative coupling reagents[8] have been investigated, that give more soluble ureas, and alternative links to the spacer[7] have been tried. Carbonate and carbamate groups appear to perform as well as succinate groups and are cleavable by ammonolysis.

After the first nucleoside is attached at suitable loading, unreacted amine sites on the support must be "capped" to prevent their reaction with nucleotide monomers during synthesis. This is normally done by acylation with acetic anhydride and catalysis with DMAP in the presence of base. Pons has stated[8] that both coupling and capping are more efficient after acid activation of the resin, the theory being that some sites on the cpg remain inactive under the basic conditions of the functionalisation step only to become activated by the first acid wash of the oligonucleotide synthesis. This is thought to be the cause of capped resins giving greater than 100% effiency for the first coupling reaction in syntheses.

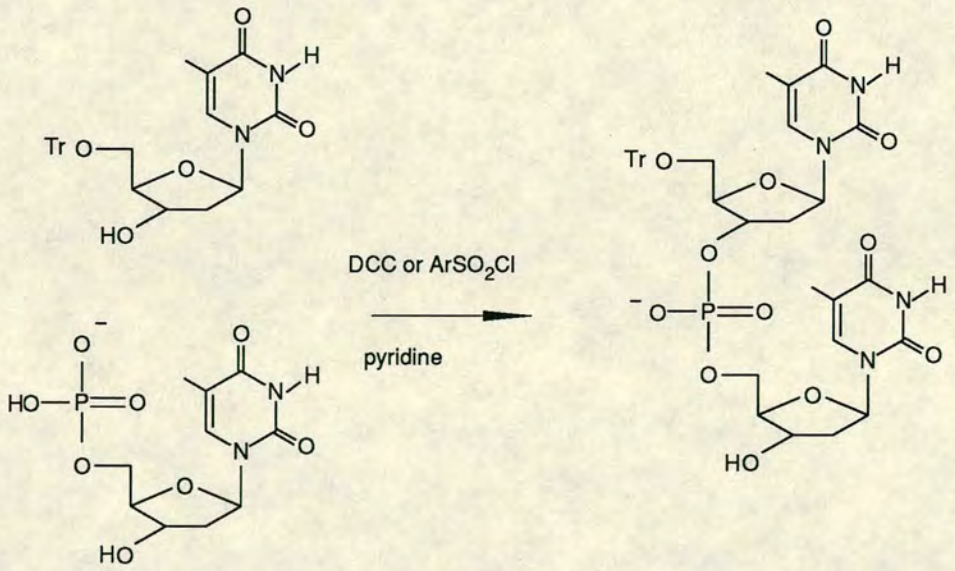
## 1.3 **Chemical Methods of Oligonucleotide Synthesis**

Four principle methods of oligonucleotide synthesis have been investigated, phosphodiester, phosphotriester, phosphite-triester and H-phosphonate. Only the latter 3 have been extensively used in solid supported synthesis.

### 1.3.1 The phosphodiester approach.

In 1956 Khorana and co-workers[9,10] showed that thymidine-5'-O-phosphate could be activated by toluene-4-sulphonyl chloride and coupled to the 3'-hydroxyl group of a 5'-protected thymidine (Scheme 3). Another condensing agent DCC was also found to be successful. The first of these reagents tends to sulphonylate the free 5'-hydroxyl in a competing reaction with condensation so a more sterically hindered sulphonyl chloride, 2,4,6-triisopropyl-benzenesulphonyl chloride has been subsequently used. This reagent cannot directly approach the 5'-hydroxyl.

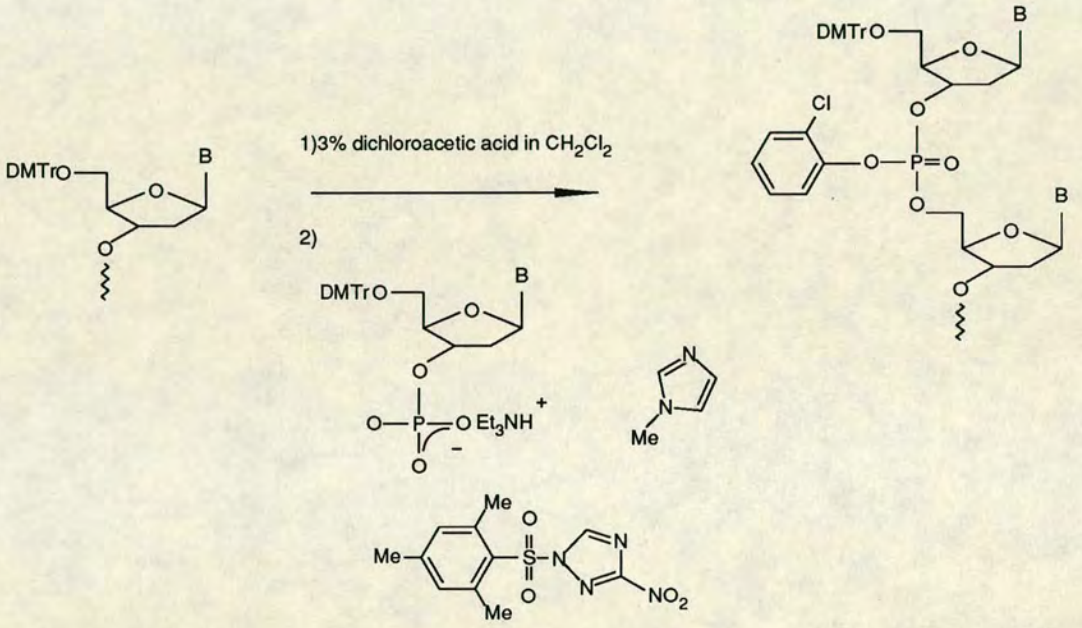
As the oligonucleotide chain length is increased the coupling reaction becomes steadily less selective due to phosphorylation of the nucleophilic phophodiester backbone. The oligonucleotide backbone, being charged, is rather insoluble in organic solvents which are used in purification in solution syntheses. Thus the purification must be carried out in polar solvents, risking loss of protecting groups due to hydrolysis. The phosphate monomers themselves are only soluble in polar solvents and cannot be purified by conventional chromatographic techniques[11]. While much pioneering synthesis was achieved by this method, it is no longer in common use.




(Scheme 3.)

1.3.2 Phosphotriester synthesis

The phosphotriester approach initially proposed by Letsinger[12] has been developed into a routine method of solid phase oligonucleotide synthesis. The 5' protected 3'-phosphodiester monomers are condensed with the 5'-hydroxyl group of the support bound oligonucleotide with the aid of an activating agent, 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole, and a catalyst, N-methylimidazole (Scheme 4).




( Scheme 4. Phosphotriester Synthesis )

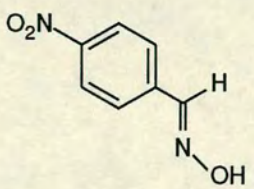
After capping unreacted 5'-hydroxyl sites with acetyl groups, the 5'-protecting group of the newly added monomer can be removed and the next monomer added(Table 1). The oligomer thus grows with an uncharged phosphotriester backbone. Several phosphate protecting groups have been investigated in this context, the aim being to allow the protecting group to be removed at the end of synthesis without any concommitant phosphate backbone cleavage. The initial syntheses employed 2-cyanoethyl protection, removable with concentrated ammonia solution, while Eckstein and Rizk introduced 2,2,2-trichloroethyl[13] later abandoned because of the difficulties in removal. The 2,2,2-trichloroethyl moiety was removed with zinc dust in 80% glacial acetic acid.

| Procedure | Reagent | Time/s |
|---|---|---|
| Wash | Pyridine | 120 |
| Wash | 1,2-dichloroethane | 120 |
| Deblock 5'-hydroxyl | Trichloroacetic acid in pyridine (3g/100ml) | 120 |
| Wash | 1,2-dichhloroethane | 120 |
| Wash | Pyridine | 120 |
| Coupling reaction | 120mg monomer, 15mg MSNT, 0.2ml 10% N-methylimidazole in pyridine | 900 |

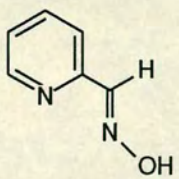
( Table 1. Phosphotriester Synthesis Cycle )

The acidic conditions may lead to depurination side reactions and a zinc/copper couple in 90% acetic acid was shown by Cook to induce loss of u.v. absorbance in the case of 4-N-benzoyl-2-deoxycytidine[14].

Reese and coworkers chose aryl groups for internucleotide protection[15], reasoning that the acidity of these groups should ensure that hydrolysis of the phosphotriester led exclusively to the loss of a phenol group without backbone cleavage. It was found[15] however that removal of these internucleotide protecting groups with strongly basic and nucleophilic reagents such as hydroxide ion caused backbone degradation. Milder nucleophilic reagents such as syn-4-nitrobenzaldoxime were therefore developed to remove aryl protection[16] (Fig. 7).



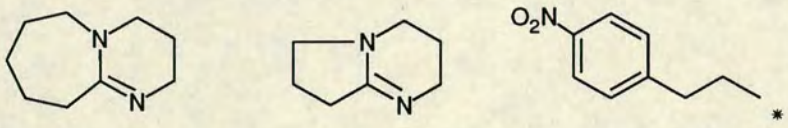

syn-4-nitrobenzaldoxime




syn-pyridine-2-aldoxime

( Figure 7. )

The initial aryl groups employed were phenyl groups, where internucleotide protection had to be stable to the mild alkaline hydrolysis of the aryloxyacetyl protection at the 5'-hydroxyl site. Where non-hydrolytic means are used to remove 5'-protection more acidic phosphate protection such as 2-chlorophenyl[16] can be employed and it is this group along with 2,5-dichlorophenyl[17] which is now commonly used in solid phase triester synthesis. Oximate ions are routinely used for their expulsion[2]. Once the backbone has been converted to a phosphodiester it is completely stable to the more stringent conditions required to remove the exocyclic amine protecting groups. The 4-nitrophenylethyl group[18] (Fig. 8), which is removed by non-nucleophilic bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Fig. 8) in a beta-elimination mechanism, has also been used for phosphate protection.




DBU DBN 4-nitrophenylethyl

( Figure 8. )

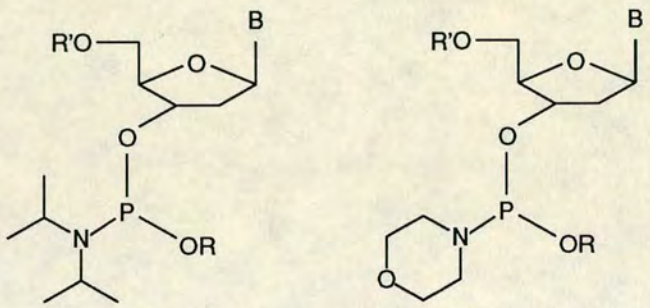
Coupling time for phosphotriester synthesis is slow by comparison to phosphoramidite and H-phosphonate chemistries but the monomers are easy to prepare and relatively stable. This stability causes them to require activation before coupling and side reactions are caused by the MSNT activator, which can react with the O(6)-atom of guanosine[19] during synthesis and lead to creation of diaminopurine bases after ammonolysis. However treatment with oximate reagent before hydrolysis of the amine protecting groups is believed to reverse this side reaction[2].

1.3.3 Phosphite-Triester Synthesis

This method was first introduced by Letsinger[20], the reactive monomer being nucleoside -3-phosphomonochloridite or monotetrazolide, with the other phosphorus protecting group being 2,2,2-trichloroethyl. These very reactive compounds couple in seconds to alcoholic hydroxyl

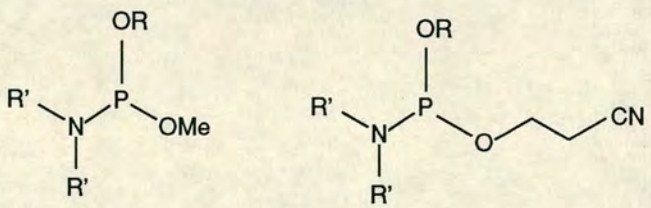
groups but are extremely unstable, being easily hydrolysed. Thus they are difficult to handle, purify and store, and reactions must be carried out at -78°C in an inert atmosphere; conditions which are unsuitable for automation and synthesis of long sequences.

Alternatives were therefore introduced and Caruthers suggested the use of 3'-O-(N,N-dimethylphosphoramidites)[21]. These compounds are more stable to hydrolysis than the phosphite triesters but are still prone to oxidation and decomposition on silica gel, making chromatographic purification impossible. With bulkier alkyl groups,[22] phosphoramidites are more stable, their stability increasing with increased steric hindrance at the phosphoramidite nitrogen[23]. N,N-Diisopropyl and morpholino phosphoramidites are now commonly used (Fig. 9).

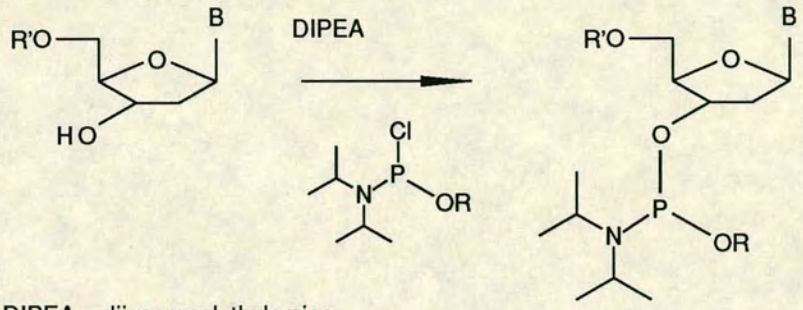



( Figure 9. )

The third group attached to the phosphorus atom, a phosphate protecting group, ensures that, after oxidation the oligonucleotide grows with a phosphotriester backbone. Early syntheses employed methoxy phosphoramidites (Fig. 10) with the methyl group being removed as the first step in deprotection by thiophenate ion. This strategy, still in common use, can lead to methylation of pyrimidines in basic conditions.[24] In addition, the thiophenol solutions which are necessary to cleave the methyl groups can be unpleasant to work with.




( Figure 10. )

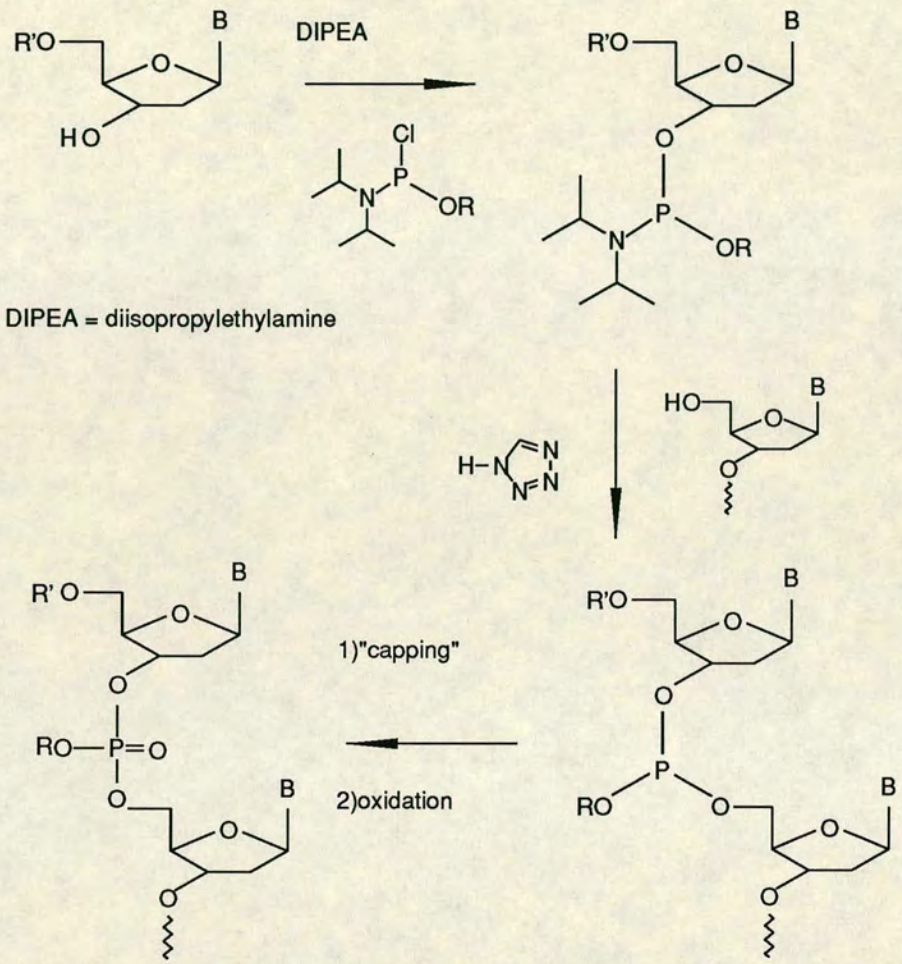



DIPEA = diisopropylethylamine

( Scheme 5. Preparation of Cyanoethylphosphoramidites)

| Operation | Reagent/solvent | Time/s |
|---|---|---|
| 1. Wash | dichloromethane | 30 |
| 2.deblock 5' | 3% trichloroacetic acid in dichloromethane | 50 |
| 3. wash | dichloromethane | 30 |
| 4.wash | anhydrous acetonitrile | 60 |
| 5.coupling step | 0.1M phosphoramidite in acetonitrile<br>0.5M tetrazole in acetonitrile | 30 |
| 6.capping step | acetic anhydride/lutidine/THF 1/1/8<br>17.6% N-methylimidazole in THF | 40 |
| 7.oxidation | 0.1M $I_2$/water/pyridine/THF<br>2/20/80 | 50 |

( Table 2.)




( Scheme 6. Phosphoramidite Synthesis )

A protecting group with none of these drawbacks is 2-cyanoethyl[25] (Fig. 10, Scheme 5). This group is rapidly removed by a beta elimination mechanism upon addition of ammonia solution, the product being the relatively unreactive acrylonitrile.

Synthesis cycle for phosphoramidite chemistry(Scheme 6, Table 2).

It is essential that the capping step is carried out before oxidation because the acylation and hydrolytic washes remove ring adducts such as tervalent phosphites and phosphoramidites which, if oxidised, become stable and lead to extensively branched products[27].

*In situ* phosphitylation has also been investigated for phosphoramidite oligonucleotide synthesis[27]. In this approach the 5'-protected nucleoside is functionalised using a di-(dialkylamine) methoxyphosphine and a di-isopropylamine tetrazolide salt activator in a pre-reaction before addition of a mixture of the nucleotide and tetrazole to the support bound oligomer.

### 1.3.4 H-Phosphonate Chemistry in Oligonucleotide Synthesis

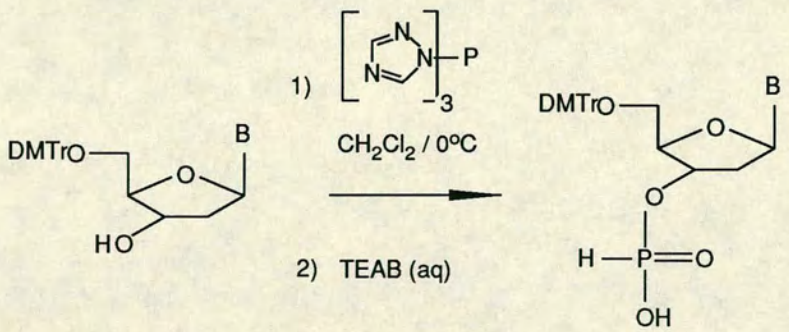
This method was originally explored by Todd and coworkers[28] in the early days of oligonucleotide synthesis. In the late 1970's Sekine and Hata used nucleoside 3'-H-phosphonates in reactions with 3'-O-protected nucleosides in oxidative phosphorylating conditions using 2,4,6-triisopropylbenzenesulphonyl chloride as a condensing agent[29].

More recently the H-phosphonate method has been adapted for automated solid phase oligonucleotide synthesis[30,31]. The coupling reaction leads to the formation of an internucleotide H-phosphonate linkage which is then oxidised to give a phosphodiester backbone. Because the H-phosphonate is stable to acidic conditions but unstable towards basic conditions, a single oxidation step is used once the entire oligonucleotide has been assembled (Table 3). The oligonucleotide is cleaved from the resin in the normal way by treatment with concentrated ammonia solution at room temperature for 90 minutes and the acyl protecting groups are removed by heating in concentrated ammonia at 55°C for 5hours.

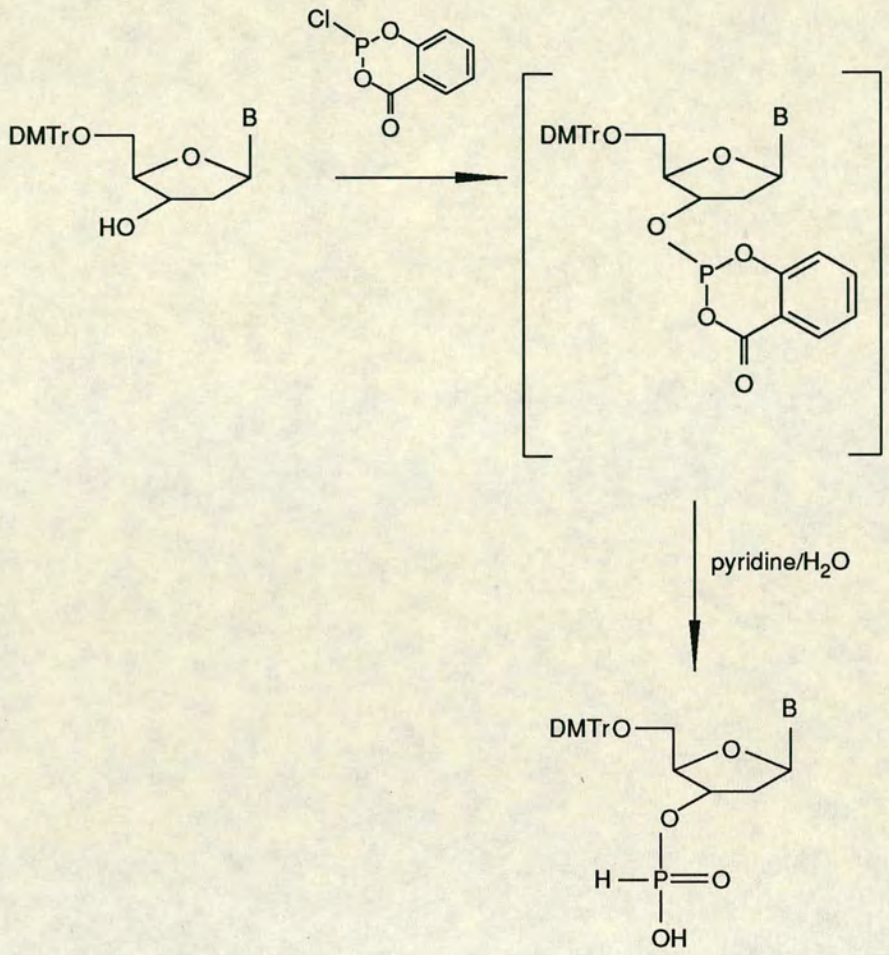
H-Phosphonate monomers are simple to synthesise by a variety of methods, one of which involves the reaction of phosphorus trichloride with triazole to give a triazolide which is further reacted *in situ* with the protected nucleoside and subsequently hydrolysed to the H-phosphonate[32] (Scheme 7). In another approach a purifiable phosphite reagent is obtained from the reaction of phosphorus trichloride with salicylic acid and this salicylate is then combined with the nucleoside and hydrolysed *in situ* to the H-phosphonate[33] (Scheme 8).

| Operation | Reagent/solvent | time/s |
|---|---|---|
| 1. wash | anhydrous acetonitrile | 45 |
| 2. deblock 5' | 2.5%dichloroacetic acid in dichloromethane | 60 |
| 3. wash | pyridine/acetonitrile 1/1 | 45 |
| 4. coupling step | 10mM deoxynucleotide H-phophosphonate<br>50mM pivaloyl chloride in pyridine/acetonitrle | 90 |
| 5. repeat 1-4 until sequence complete | | |
| 6. deblock 5' | 2.5%dichloroacetic acid in dichloromethane | 45 |
| 7. oxidise | a)0.1M I2 in pyridine/N-methylimidazole/water-<br>/tetrahydrofuran 5/1/5/90 | 90 |
| | b)0.1M I2 in triethylamine/water/tetrahydrofuran<br>5/5/90 | 150 |

( Table 3. Phosphonate Synthesis Cycle)

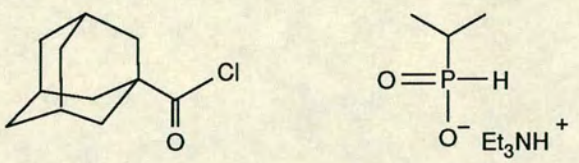



( Scheme 7. )




( Scheme 8. )

The coupling agents in H-phosphonate oligonucleotide synthesis are acid chlorides which react with the H-phosphonates to give acylated reactive intermediates. Pivaloyl (trimethylacetyl) chloride was initially used but this reagent degrades upon storing and has been shown to acylate the internucleotide phosphonate linkage[34]. An alternative reagent, adamantoyl carbonyl chloride (Fig. 11), is therefore now commonly used in commercial synthetic protocols[35].




( Figure 11.)

A capping step[35] has also been introduced into the H-phosphonate oligonucleotide synthesis cycle using isopropyl phosphite (Fig. 11) with the adamantoyl chloride activating reagent.

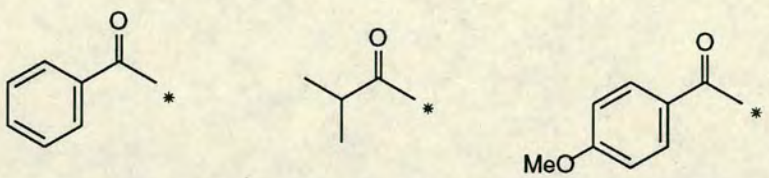
H-phosphonate intermediates provide a convenient route to a number of modified oligonucleotides. Phosphorothioates[36], phosphoramidates[37,38] and phosphotriesters[39] can all be produced by chemical treatment of the H-phosphonate backbone.

## 1.4 **Protecting groups for Oligonucleotide Synthesis**

Two classes of protecting groups are required during conventional solid supported oligonucleotide synthesis.The first is temporary protection for the 5'-hydroxyl group of the added monomer during the condensation reaction. This protection is then removed during the synthesis to provide the next phosphorylation site. The second variety of protection is semi-permanent blocking of any nucleophilic moiety capable of interfering with the chain extension procedure. Thus exocyclic amino functions, the 2'-hydroxyl of ribonucleosides and in a few cases the imide nitrogen of the heterocyclic base require protection which remains in place throughout the synthesis.

### 1.4.1 Amino Protection

The N(6) amino group of adenosine, the N(4) amino group of cytosine, and the N(2) amino group of guanosine all require protection during synthesis. This is commonly acyl protection which is removed after completion of synthesis by mild alkaline hydrolysis with concentrated aqueous ammonia solution at 55°C overnight. Benzoyl groups are commonly used for adenosine and cytosine, while isobutyryl-protection is used for guanosine, as benzoyl groups are too slow in hydrolysing and often lead to solubility problems[40](Fig. 12). p-Methoxybenzoyl(anisoyl) protection is also common (Fig. 12). As the acid chloride is slightly more reactive and the protecting group slightly more labile than benzoyl, it can be used on both exocyclic amino functions and imido ring nitrogens.

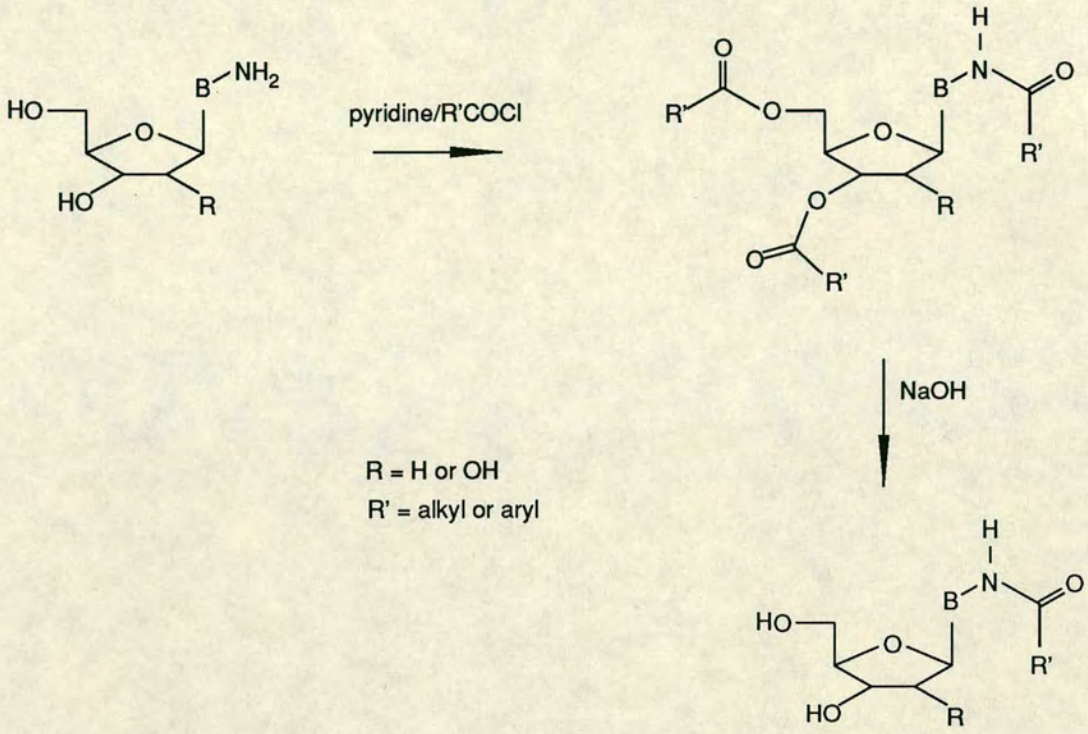



Benzoyl Isobutyryl 4-Methoxybenzoyl (Anisoyl)

( Figure 12. )

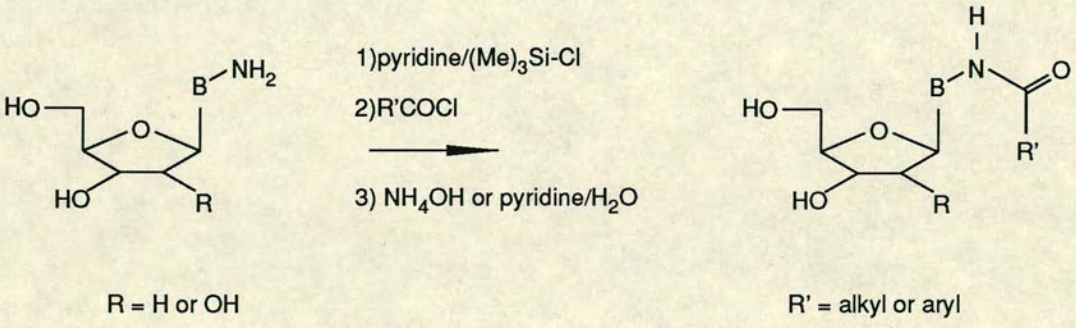
Acyl protection is commonly introduced by one of two methods.

(1) Traditionally per-acylation of the nucleoside followed by hydrolysis of the ester linkages with dilute sodium hydroxide solution (saponification) was employed[41] (Scheme 9).




( Scheme 9. Saponification route to protect amino functions )

(2) More recently a transient hydroxyl protection method,employing trimethylsilyl groups, has been introduced by Gaffney and Jones[42] (Scheme 10). This reduces protection to a one pot reaction.

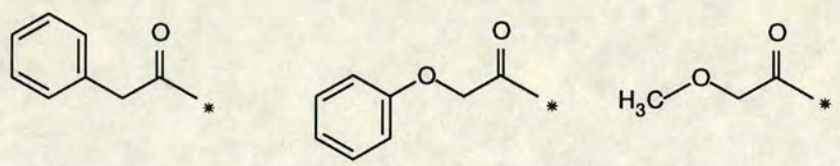



( Scheme 10. Transient methodology for the introduction of protecting groups )

Selective N -acylating reagents have also been designed to avoid the saponification route, including 2-(chloromethyl)-4-nitrophenyl benzoate[43], O-ethyl-S-benzoyl dithiocarbonate[44], p-nitrophenyl benzoate / hydroxybenzotriazole[45] and pentaflorophenyl benzoate[46]. However the active ester method is only effective for the protection of N(4) in cytosine. With the other bases N-acylation is slow and concommitant O-acylation occurs[42] .

The phenylacetyl group[40] has been used to protect the N(2) of guanosine. The group is easily introduced by the transient protection methodology, has good solubility and is significantly more labile to aqueous ammonia than the 2-N-isobutyrylguanine protecting group.

Even more labile acyl protecting groups (Fig. 13) introduced by Teoule and coworkers can be removed without the need to heat in ammonia solution at all. It has been reported that phenoxyacetyl protection for guanosine and adenosine and isobutyryl protection for cytosine can be hydrolysed at room temperature in 29% ammonia solution in less than 4 hours[47]



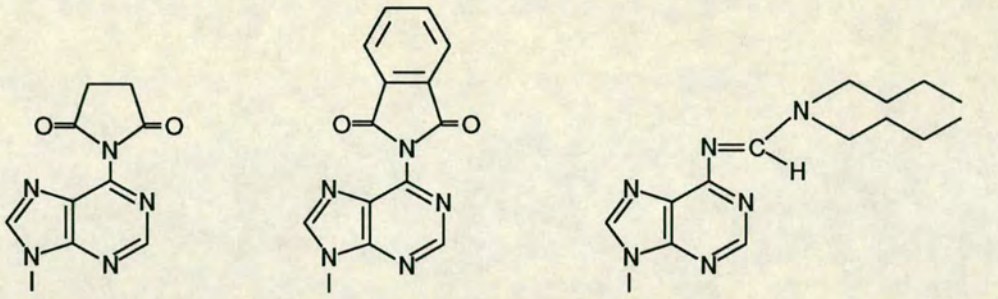

Phenylacetyl Phenoxyacetyl Methoxyacetyl

( Figure 13. )

In the case of adenosine it has been shown that N(6) benzoyl protection promotes depurination during acid treatment[48,49]. Cyclic diacyl groups have been shown to slow this side reaction considerably[50]. Succinyl and phthaloyl groups (Fig. 14) are the most successful, N(6) succinyl protection increasing the half life of adenosine in 80% acetic acid at 70°C by 7-fold in comparison to benzoyl protection. In addition, where depurination is at its worst, at the 3' end of the oligonucleotide, succinyl groups can be introduced to the 3'-nucleoside at N(6) and 3'-OH in a one pot reaction. Cyclic diacyl groups are not stable in the presence of fluoride ions[50].

An alternative depurination-resistant protecting group has been suggested by Froehler and Matteuci[51] (Fig.14). Dialkylformamidines have been shown to increase stability of 2'-deoxyad-

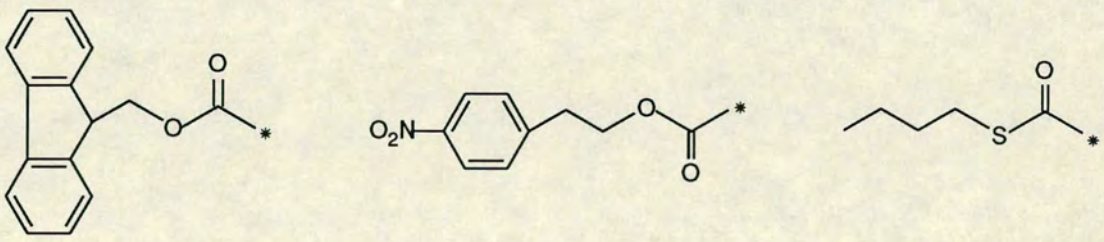
enosine to 80% acetic acid by up to 24 fold relative to benzoyl protection. The di-n-butyl formamidine group is removed under the "standard " basic hydrolytic conditions and introduced by amino- specific reaction of the nucleoside with di-n-butyl formamide dimethylacetal.



6-N-Succinyladenine 6-N-Phthaloyladenine 6-N-(di-n-Butyl-formamidine)-adenine

( Figure 14. )

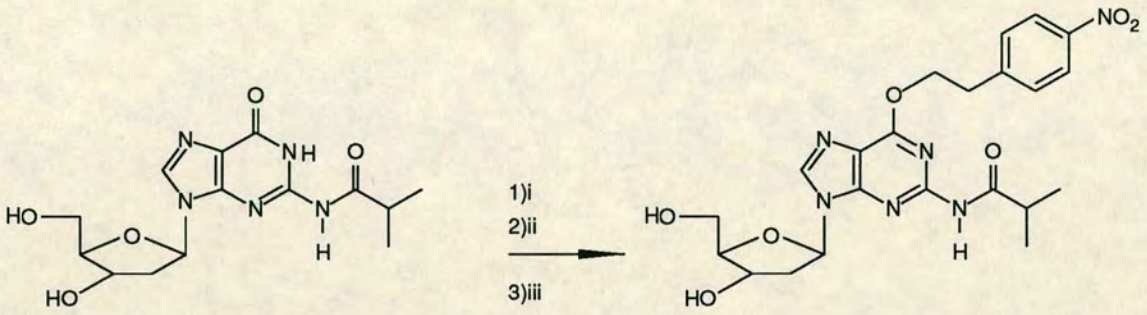
The use of amine protecting groups which can be removed nonhydrolytically, by treatment with non-nucleophilic bases in a beta elimination mechanism have also been reported. Pfleiderer and coworkers employed the 4-nitrophenylethylcarbamate group (Fig.15) to protect the exocyclic amine groups of the bases. The group was introduced by N-specific acylation of cytosine using 1-(4-nitrophenylethoxycarbonyl)-benzotriazole and with the chloroformate and transient protection methodology to the N(6) amino group of adenine[18]. The use of the 9-fluorenylmethoxycarbonyl group has been reported[52,53] for the protection of the N(4) site of deoxycytidine in phosphoramidite synthesis of phosphate-methylated dinucleotides (Fig. 15).



9-Fluorenylmethoxycarbonyl 4-Nitrophenylethoxycarbonyl n-Butylthiocarbonyl

( Figure 15. )

Hata and coworkers have suggested the use of the butylthiocarbonyl[54] group (Fig. 15) for the protection of the N(2) amino group of guanine and also the amido O(6)-position, which can take part in phosphorylation reactions in synthesis but is not commonly protected. The group is removed by basic hydrolysis and is stable to acid and fluoride treatment.



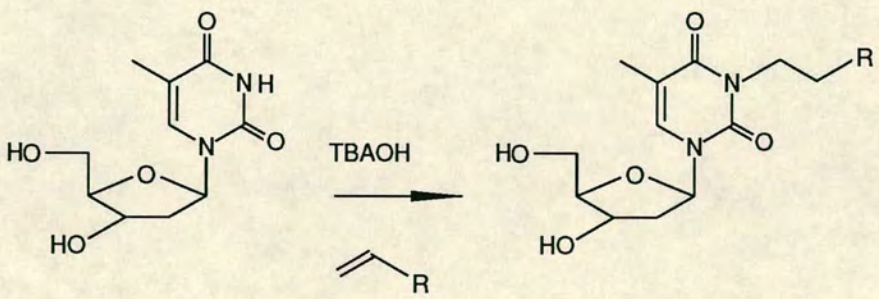

i = trimethylsilyl imidazole

ii = 4-nitrophenylethanol, triphenylphosphine, diethylazodicarboxylate

iii = 1M pyridinium hydrofluoride in pyridine

( Scheme 11. )

Other groups used to protect amido-functions within the heterocyclic rings of the bases include acyl functions (eg. methoxybenzoyl) and alkyl groups such as cyanoethyl and nitrophenylsulphonylethyl[55,56]. The latter type of protection is introduced at nitrogen by a Michael addition (Scheme 11) or at the oxygen atom of the amido function by a Mitsonobu reaction (Scheme 12).




TBAOH = tetrabutylammonium hydroxide

R = CN or 4-nitrophenylsulphonyl

( Scheme 12. )

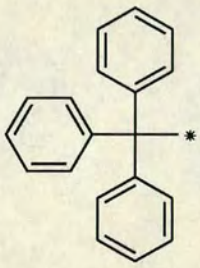
The 4-nitrophenylethyl group has also been employed in this way[18] and can be removed in a beta elimination mechanism with strong non-nucleophilic bases.

The amide ring proton is normally only nucleophilic enough to cause significant side reactions in the case of uridine, and then only in phosphotriester synthesis. The N(3) atom of uridine maybe protected by a methoxybenzoyl group.

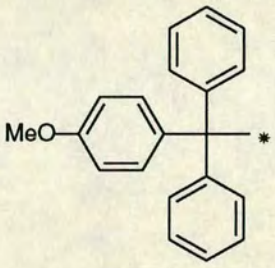
1.4.2 Temporary 5'-Hydroxyl protection.

The key requirements of temporary protecting groups are that they must be completely stable during the condensation step and then completely removable thereafter without any concommitant cleavage of permanent protection at amino, hydroxyl, or phosphorus. Selective addition of the protecting group to the 5'-hydroxyl group during synthesis of the monomer is also important.

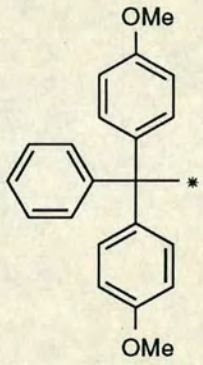
The most successful and widely used groups for this purpose are triarylmethyl ethers[57](Fig. 16).



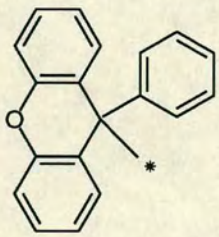
Trityl (triphenylmethyl)



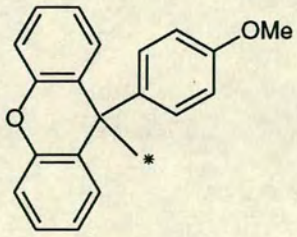

4-Monomethoxytrityl




4,4'-Dimethoxytrityl



Pixyl (9-phenylxanthen-9-yl)



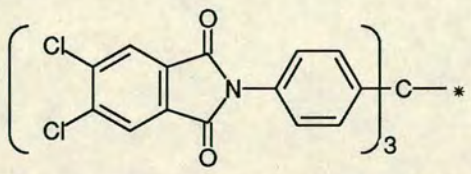

Moxyl [9-(4-methoxyphenyl)xanthen-9-yl]

( Figure 16. )

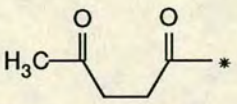
These groups are introduced by reaction of triarylmethyl halides with the alcohol in the presence of base and are cleaved quickly by acidolysis (the 4,4'-dimethoxytrityl group is removed in less than 30 seconds by 3%trichloroacetic acid in dichloromethane[2]). The strongly coloured cations released during acid cleavage allow the amount of oligonucleotide present to be measured spectrophotometrically at 495nm and therefore the stepwise efficiency of the coupling reaction can be monitored during the synthesis.

The 9-phenylxanthen-9-yl (Pixyl) group[58] (Fig. 16) has the advantage of giving crystalline nucleoside products[2] but 4,4'-dimethoxytrityl protection is the most commonly employed, as 4,4'-dimethoxytrityl chloride is readily available commercially, whereas the 9-phenylxanthen-9-yl chloride is not.

Acid washes during oligonucleotide synthesis can lead to depurination side reactions[49,51] and so removal of the 4,4'-dimethoxytrityl group with Lewis acids such as zinc bromide have been investigated[59,60]. However the rate of the cleavage reaction then depends upon oligonucleotide chain length and insoluble complexes can be formed if traces of pyridine are present. Hence zinc bromide is unsuitable for solid phase synthesis. A protected triarylmethyl protecting group has been employed which does not require acid deprotection. The 4,4',4''-tris(4,5-dichlorophthalimido)-trityl group[61] (Fig 17) is removed by cleavage of the phthaloyl groups with hydrazine. Levulinyl protection[62] (Fig. 17) is also removed by hydrazine but its introduction at the 5'-OH is insufficiently specific for everyday use.




CPTr



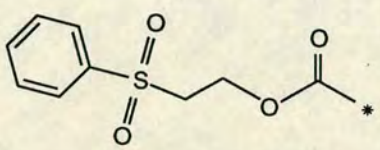

Levulinyl

( Figure 17. )

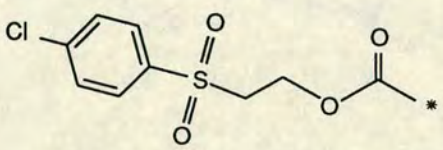
The dibromomethylbenzoyl group (DBMB)[63] has been employed as a 5'-OH protecting group removable under completely neutral conditions. Oxidation first converts the dibromo-derivative

to a formyl moiety and the altered group is then rapidly cleaved by addition of morpholine.

5'-Hydroxyl protection removable by a beta-elimination mechanism in the presence of non-nucleophilic bases has also been proposed. 2-Phenylsulphonylethyl[64] (Fig. 18) and 2-(4-chlorophenyl)-sulphonylethyl groups[65] (Fig. 18), introduced as carbonates by reaction of the 5'-hydroxyl group of the sugar, withthe appropriate chloroformates, are cleaved by treatment with triethylamine. The 9-fluorenylmethoxycarbonyl group has been used in both oligodeoxynucleotide synthesis[66,67] and oligoribonucleotide synthesis[68] to protect the 5'-hydroxyl group of the sugar moiety.



2-Phenylsuphonylethoxycarbonyl



2-(4-chlorophenyl)-sulphonylethoxycarbonyl

( Figure 18. )

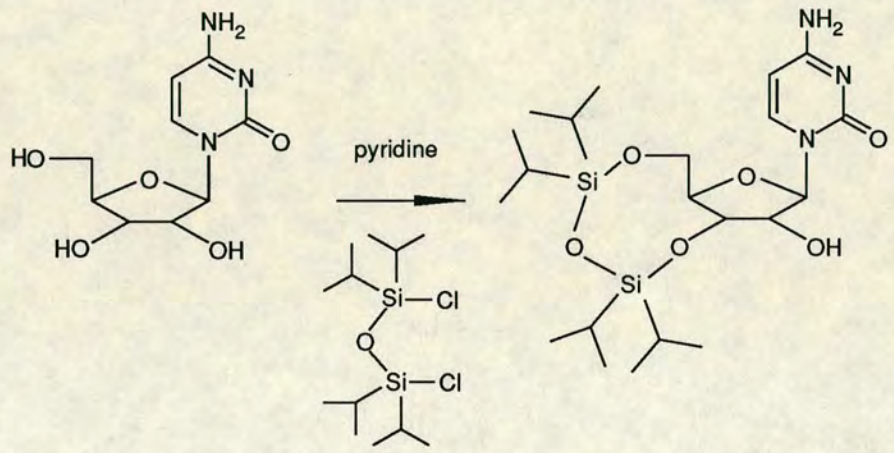
1.4.3 Protection of the 2'-Hydroxyl Group of Ribose in Oligoribonucleotide Synthesis.

Protection of the 2'-hydroxyl function of ribonucleosides has been found to be essential in oligonucleotide synthesis. Ideally, the protocol should be such that all other protection can be removed before the 2' -group is cleaved. This will generally prevent any 3'-2' migration of the internucleotide phosphate link occurring and will also prevent any enzymic cleavage of the oligoribonucleotide on long term storage.The movement of the internucleotide link from 5'-3' or 3'-2', leading to chain cleavage, has been observed in strongly acidic media and in all basic conditions for phosphotriester linkages[11]. Thus where acyl base protection is used, 2'-hydroxyl protecting groups must be stable to mild alkaline hydrolysis. In general acetals and ethers are found to be stable to such conditions.

Introduction of any group specifically at the 2'-hydroxyl site is difficult because of the need to differentiate it from the other secondary hydroxyl at the 3' position. Many reactions are found to occur more readily at the 2'-hydroxyl site than the 3'-hydroxyl site if the 5'-hydroxyl group is

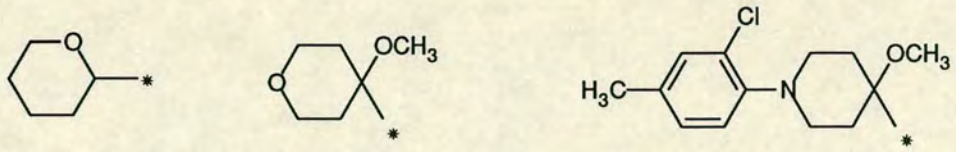
blocked. Thus triisopropylsilyl groups can be introduced in 60% yield to 2'-hydroxyl of 5'-O-(4,4''-dimethoxytrityl)-4-N-benzoylcytidine[69].

A group which selectively and simultaneously protects at both 5' and 3' sites has been introduced by Markiewicz[70] (Scheme 13). Preparation of the 3',5'-O-tetraisopropyldisiloxane-1,3-diyl nucleoside derivative allows the 2'- hydroxyl to be reacted with excess protecting reagent in forcing conditions. This silyl group is stable except in strongly acidic or strongly basic media and is removed by the action of fluoride ions.Thus almost any group stable to fluoride ions can theoretically be attached selectively to the 2'-hydroxyl position.




( Scheme 13. )

The tetrahydropyranyl group (THP) (Fig. 19) for the protection of alcohols introduced by Parham and Anderson[71] was the first acetal link investigated for RNA synthesis[72,73,74]. The group, put on with dihydropyran and acid catalysis and removed with dilute acid, has good stability but is chiral, leading to the formation of diastereomers when attached to optically active alcohols such as nucleosides[75]. A symmetrical acetal, 4-methoxytetrahydropyran-4-yl(MTHP) (Fig. 19), was therefore designed and synthesised by Reese and coworkers[76]. It is generally introduced to the tetraisopropyldisiloxyl nucleoside and is removed from the oligonucleotide with hydrochloric acid at pH 2 in 1hour after all acyl protection has been removed. It has been employed by Gait and coworkers[68] with 5'-9-fluorenylmethoxycarbonyl and by van Boom using 5'-levulinyl protection[62]. However it has been shown that some loss of MTHP occurs during the acidolysis of triarylmethyl




THP MTHP CTMP

( Figure 19. )

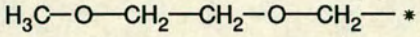
protecting groups at the 5'-hydroxyl position[77] and so 1-[-2-chloro-4-methylphenyl]-4-methoxy-piperidin-4-yl(CTMP)[78] (Fig. 19) has been introduced, which becomes protonated and therefore more stable at the low pH of 5'-acidolysis, while being unprotonated and easily cleaved at slightly higher(2.5) pH. Addition of electron withdrawing groups stabilises acetals to acid hydrolysis[79].

An alternative approach to oligoribonucleotide synthesis introduced by Ogilvie and coworkers[69] maintains 5'-O-(4,4''-dimethoxytrityl) protection but uses a tert.butyldimethylsilyl ether(TBDMS) (Fig. 20) group for 2'-hydroxyl protection.This moiety is introduced by direct reaction of 5'-protected nucleoside with TBDMS-chloride in the presence of imidazole. The mixture of 2' and 3' isomers produced can be separated chromatographically. The TBDMS group is removed with fluoride ions after all other protection has been cleaved[80]. This strategy for oligoribonucleotide synthesis has now been widely adopted and is standard on some commercial oligonucleotide synthesisers. A 70mer has been made[69] in addition to very large amounts of several dodecamers for NMR work[81].


Tert-butyldimethylsilyl (TBDMS) Triisopropylsilyl

( Figure 20. )

Other ether 2'-hydroxyl protecting groups used include methoxybenzyl[82] (Fig. 21), o-nitrobenzyl[83] (Fig. 21), and methoxyethoxymethyl[84] (Fig. 21). The first is removed oxidatively, while o-nitrobenzyl is cleaved by uv photolysis and treatment with zinc bromide catalyses the cleavage of the methoxyethoxymethyl group.



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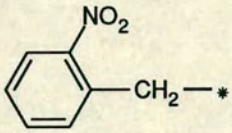
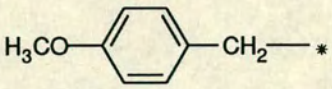


2-Nitrobenzyl



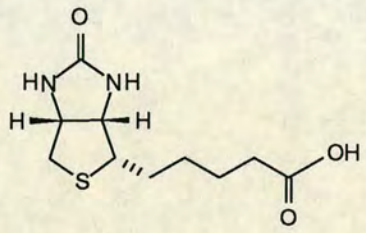
4-Methoxybenzyl

( Figure 21. )

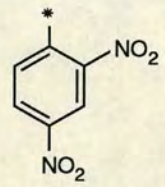
## 1.5 **Fluorescent Labelling of Oligonucleotides**

Synthetic oligonucleotides are of vital importance in biological applications as sequence specific probes and as primers for DNA sequencing.For both applications the oligonucleotides must be labelled for identification. Commonly labelling is by enzymatic addition of a radioactive $^{32}P$ phosphate group using the enzyme T4 polynucleotide kinase and gamma ATP, but more recently much interest has been shown in methods of incorporating non-radioactive labels and therefore avioding the problems of working with radioactivity, which can be hazardous to health and expensive. A further problem with radioactive labelling is that the lifetime of the label is short because of $^{32}P$ decay.

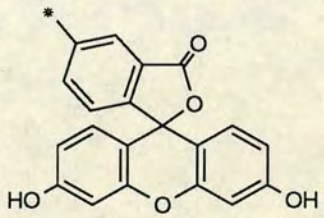
The labelling of oligonucleotides with biotin[85] (Fig. 22), an immunogenic tag which binds to eggwhite avidin and bacterially derived streptavidin, and with other groups such as dinitrophenyl (Fig. 22), also detected immunogenically, or fluorophores (Fig. 22) detected by laser induced fluorescence[86] have been investigated. Enzymes such as horseradish peroxidase and alkaline phosphatase have also been used to label oligonucleotides. In these cases detection is by colorimetry or fluorescence [87,88].



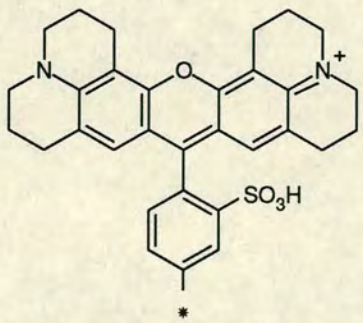
Biotin



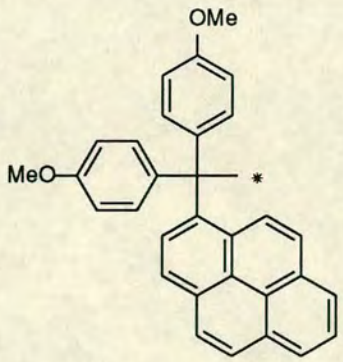
2,4-Dinitrophenyl



Fluorescein



Texas red

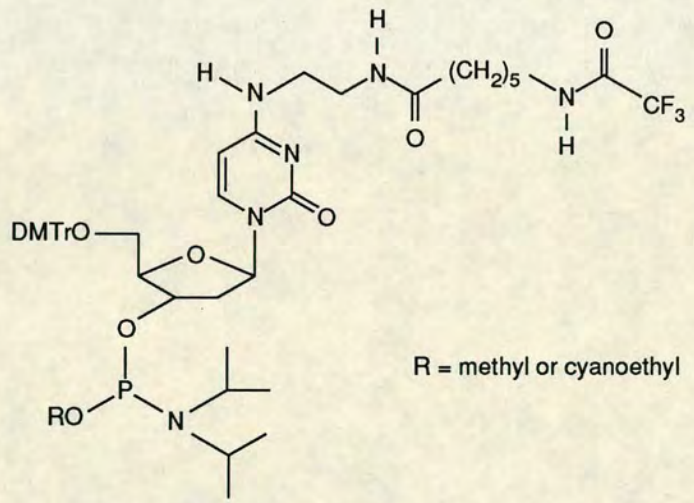


1,1-bis-(4-methoxyphenyl)-1-pyrenylmethyl (BMPM)

( Figure 22. )

With the exception of the BMPM[89] group (Fig. 22) which is a 5-hydroxyl group similar to the 4,4'-dimethoxytrityl group, these chemical labels are introduced by reaction of their electrophilic derivatives with nucleophilic primary aliphatic amines.Thus the oligonucleotide must be modified to carry a primary aliphatic amine function.This is usually done so that the amine is added during routine oligonucleotide synthesis. Two general methods of achieving this are either to modify a nucleotide to carry a protected amine or to produce a simple nucleotide analogue with a protected amine function. Once synthesis is complete all protection can be removed and the oligonucleotide can be reacted with the activated label.

Modification of the 4-amino group of cytosine to eventually produce a 4-N-[-N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5-methyl-2'-deoxycytidine[88] can be achieved by production of a 4-triazolocytosine[90] derivative followed by displacement with ethylenediamine[91,92] and reaction of this compound with N-hydroxysuccinimidyl N-trifluoroacetyl-6-aminocaproate. After conversion to a N,N-diisopropylmethoxyphosphoramidite, this monomer (Fig. 23) can be used in standard oligonucleotide synthesis.

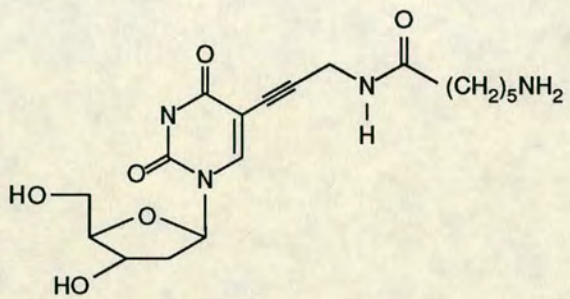



( Figure 23. )

A similar displacement approach has also been adopted by Sproat and coworkers[93] to put an aminopentyl linker at the same position. The 5',3'-O- silylated nucleoside is reacted first with mesitylene sulphonyl chloride which is successively displaced by o-nitrophenol and diaminopen-

tane. The free amine is then protected with a trifluoroacetyl group and the silyl groups are removed.

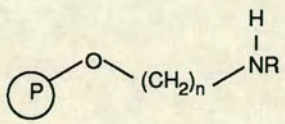
Amine groups have also been introduced by C(5) modification of deoxyuridines[94] (Fig. 24). In addition, 8-aminohexyladenosine triphosphate (available fom Sigma) has been added enzymatically to oligonucleotides which can then be reacted with dinitrofluorobenzene[95]. Primary aliphatic amines have also been attached to the N(4) amino groups of cytosine bases in polynucleotides by bisulphite catalysed transamination reactions[96].




( Figure 24. )

Substitution of an amine function for the 5'-primary sugar hydroxyl has been employed[86]. When protected at the amine site by trifluoroacetyl group, the resulting 5'-amino-2',5'-dideoxynucleoside can be phosphitylated and used as the final 5'-end base in an oligonucleotide synthesis. After deprotection, the amino-group is reacted with fluorescent dyes. These oligonucleotides form the basis of an automated sequencing scheme[97].

The nucleotide analogue technique, where a simple aliphatic amino-alcohol is converted to a monomer suitable for oligonucleotide synthesis (Fig. 25), has been employed in a number of ways.




P = phosphodiester, phosphoramidite, phosphonate

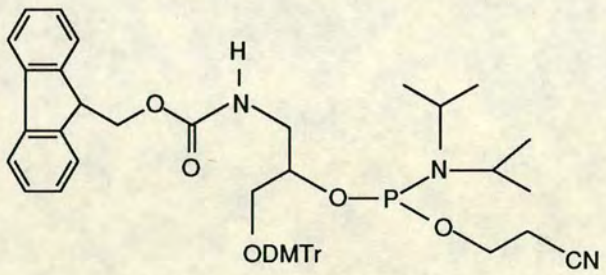
NR = protected amine function

n = 2 - 10

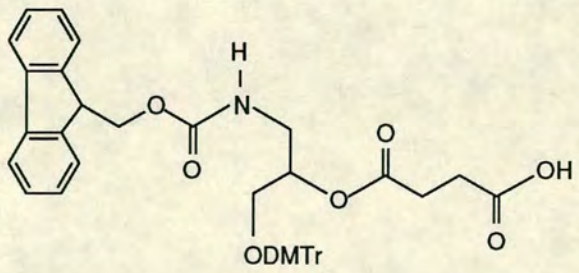
( Figure 25. )

In general an aminoalcohol is protected at the amine site and then converted to the phosphoramidite, phosphodiester or phosphonate. Amino alcohols used range in carbon chain length from 2 to 10 ( *ie* aminoethanol[98] through aminopropanol[99] to aminodecanol[100]). The following groups have been employed to protect the primary amine: phthaloyl[100]. trifluoroacetyl[98c], (removed by alkaline hydrolysis), 9-fluorenylmethoxycarbonyl[98d,100] (hydrolysis or beta elimination) and monomethoxytrityl[98b,99] (removed by acidolysis). Oligonucleotide synthesis has been by phosphotriester[98b], phosphoramidite[98.99] and alkylphosphonate[100] methodology. All these methods are limited to single label addition. However a commercially available, protected aminodiol monomer (Fig. 26) now allows multiple label addition[101] The equivalent O-succinate can be prepared and thence be used to label at the 3'-end of the oligonucleotide[102] (Fig. 27).

All the above routes require post deprotection solution phase reactions between the amino oligonucleotide and the label. While allowing a wide variety of labels to be used, this is a time consuming and sometimes low yielding process. Recently a method whereby a biotinylated monomer is added to the 5' end of the oligonucleotide has been reported[103].




( Figure 26. )



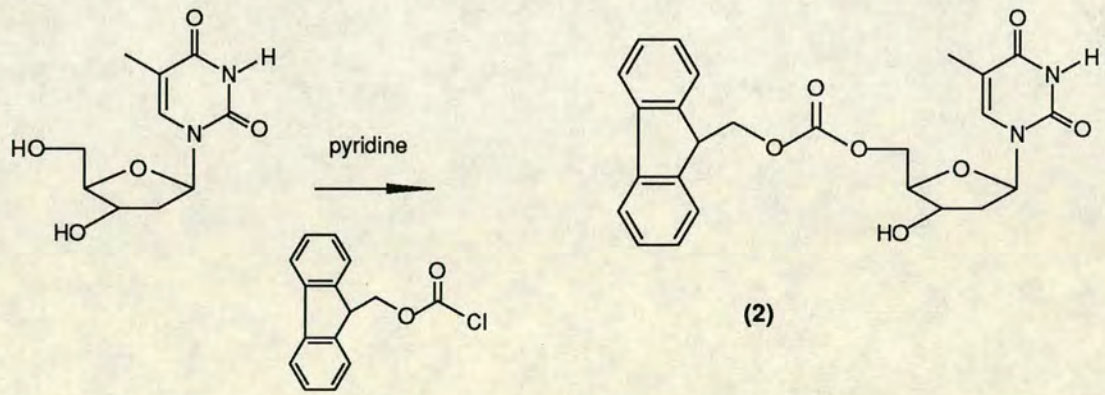

( Figure 27. )

# Results and Discussion

## 2.1 5'-O-(9-Fluorenylmethoxycarbonyl) Ribose Protection in Phosphotriester synthesis.

Solid phase synthesis has been dominated by the use of acid labile 5'-hydroxyl protecting groups, popular because they are removed in acid conditions with no risk of breaking the 3'-succinyl ester link to the support or of removing acyl exocyclic amine protection. The aim of this project was to apply beta-eliminating groups especially 9-fluorenylmethoxycarbonyl as temporary 5'-hydroxyl protection removable with non-nucleophilic bases. This might allow use of acid sensitive bases in synthesis or of permanent acid labile protection elsewhere in the oligomer , eg acetal protection at the 2'-hydroxyl in the oligoribonucleotide series.

The 9-fluorenylmethoxycarbonyl (FMOC) group introduced by Carpino[104] and developed as a group for the temporary protection of alpha amino groups during peptide synthesis by Sheppard[105], was first introduced into oligonucleotide synthesis for 5'-hydroxyl protection by Chattopadhyaya and coworkers[106]. 5'-O-(9-Fluorenylmethoxycarbonyl)-thymidine compound (**2**) was prepared by reaction of thymidine with 9-fluorenylmethyl chloroformate in dry pyridine (Scheme 14).

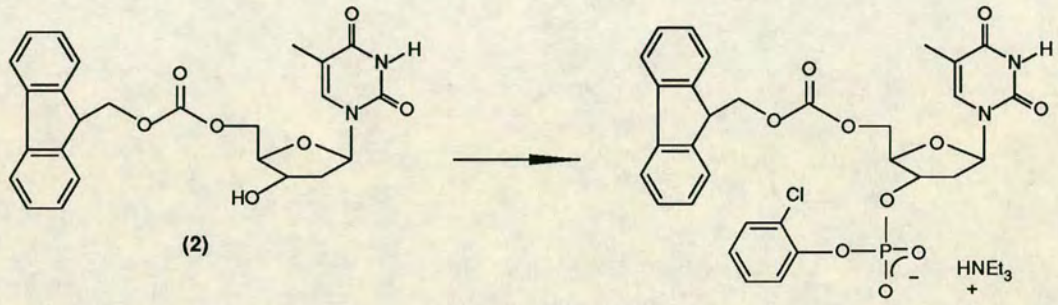



( Scheme 14. )

The crystalline product of this reaction was tested for stability to common solvents and non-nucleophilic bases. The compound was found to be stable for several hours at ambient temperature to ethanol, methanol, acetonitrile, chloroform, pyridine, dichloromethane and 1,2-dichloroethane. 5'-(9-Fluorenylmethoxycarbonyl)-thymidine was also stable in freshly distilled DMF but decomposed when dissolved in SLR grade DMF, presumably due to the presence of

primary amine contaminants. Hence solvents of this type should be avoided when using the 9-fluorenylmethoxycarbonyl protecting group. The FMOC group was cleaved from the 5'-O-(9-fluorenylmethoxycarbonyl)-thymidine nucleoside by 10% DBU in acetontrile instantaneously and by triethylamine (10% in acetonitrile v/v) in 15 minutes (monitoring by t.l.c).

5'-O-(9-Fluorenylmethoxycarbonyl)-thymidine-3'-O-(2-chlorophenylphosphate) compound(**3**) was prepared (Scheme 15) and used in a phosphotriester DNA synthesis of the deoxytetramer $(T)_4$ synthesis (**a**) ( Table 4 ), using a "home made" semi-automatic synthesiser.




(2) (3)

( Scheme 15. )

| Procedure | Reagent | Time/s |
|---|---|---|
| Wash | Pyridine | 120 |
| Wash | 1,2-dichloroethane | 120 |
| Deblock 5'-hydroxyl | 10% DBN in pyridine | 180 |
| Wash | 1,2-dichloroethane | 120 |
| Wash | pyridine | 120 |
| Coupling reaction | 20mg monomer, 15mg MSNT, 10% N-methylimidazole in pyridine (0.2ml) | 900 |

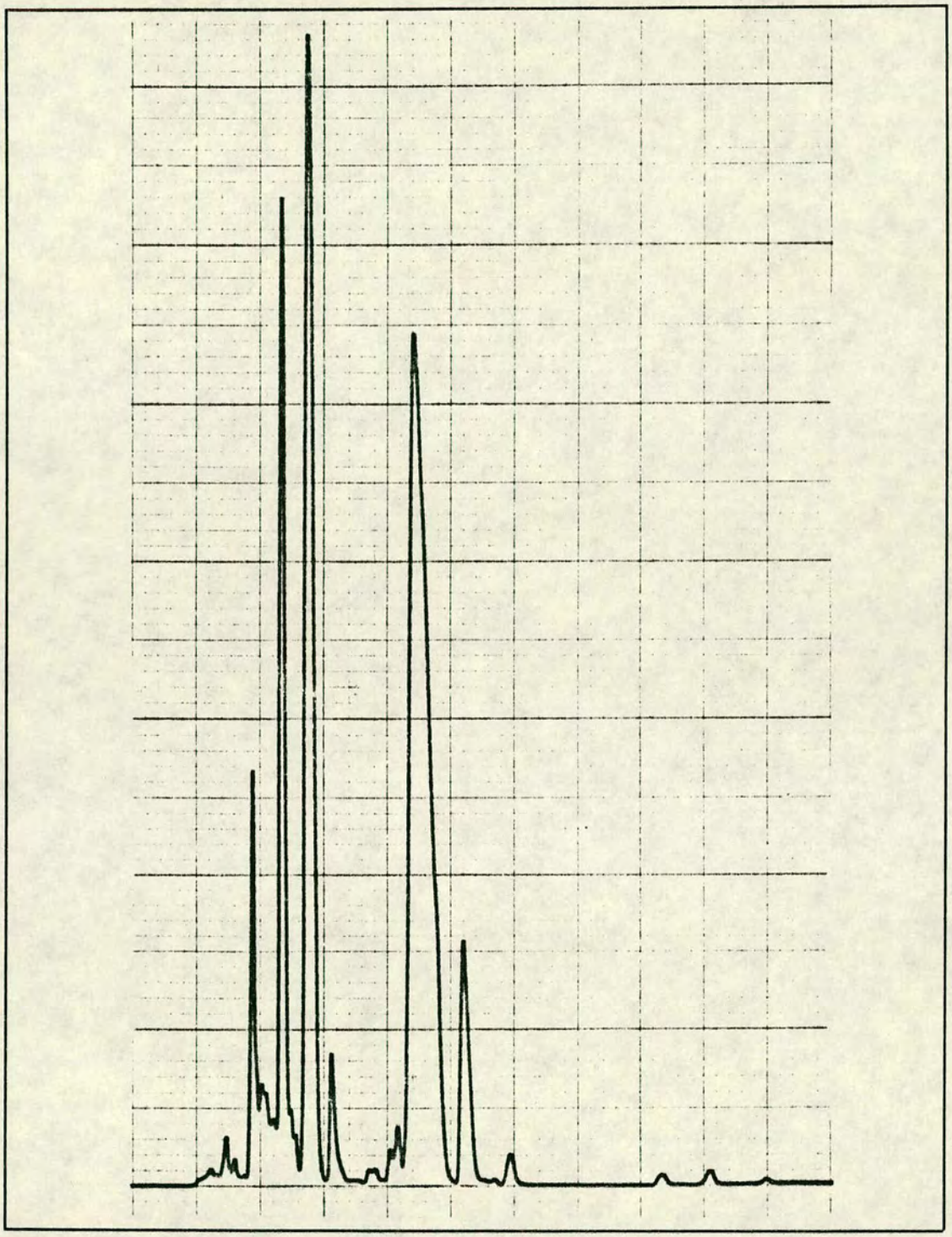
( Table 4. )

| Procedure | Reagent | Time/s |
|---|---|---|
| Wash | Pyridine | 120 |
| Wash | 1,2-dichloroethane | 120 |
| Deblock 5'-hydroxyl | Trichloroacetic acid in pyridine (3g/100ml) | 120 |
| Wash | 1,2-dich loroethane | 120 |
| Wash | Pyridine | 120 |
| Coupling reaction | 120mg monomer, 15mg MSNT, 0.2ml 10% N-methylimidazole in pyridine | 900 |

( Table 5.)

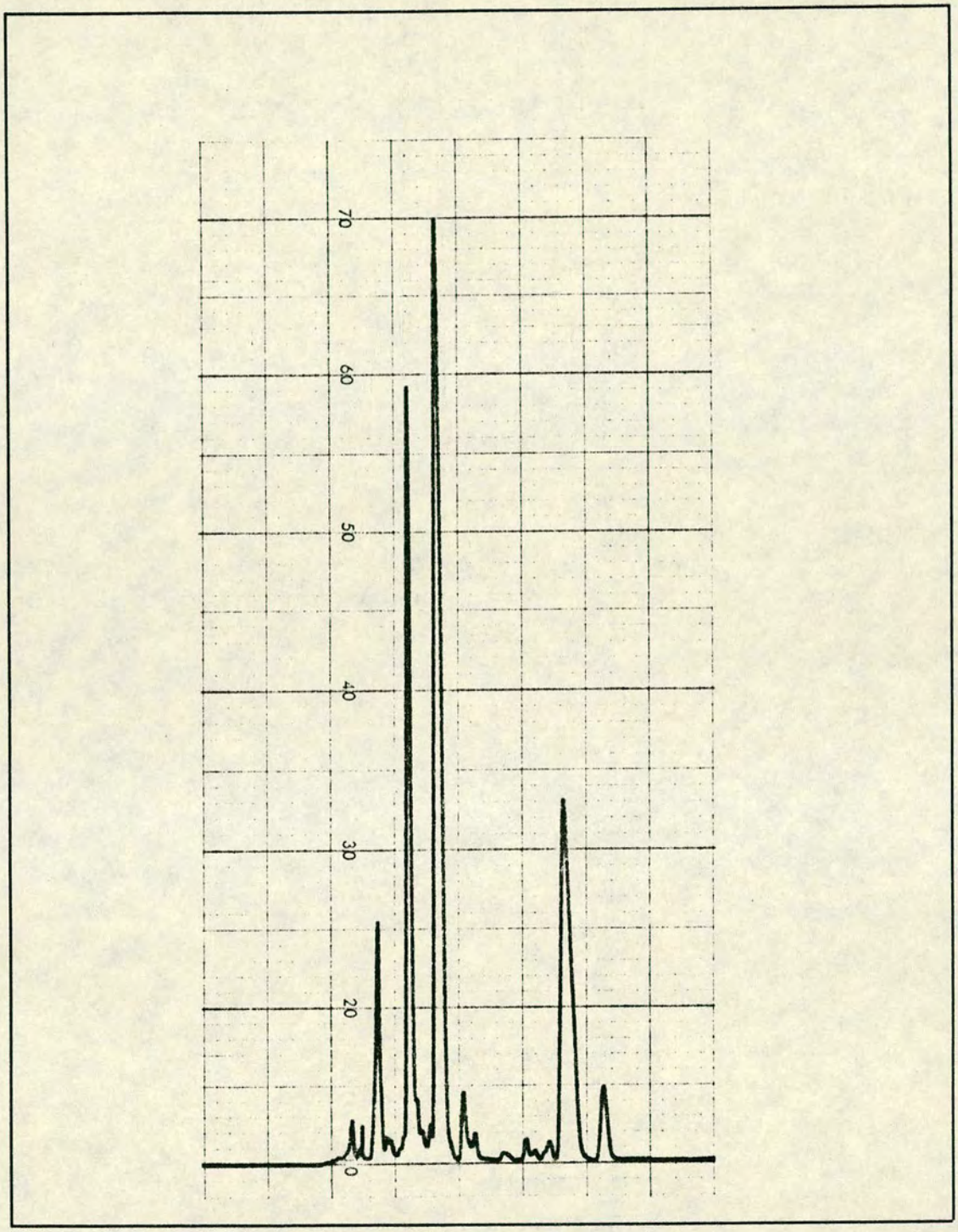
Long chain alkylamine controlled pore glass, functionalised with 5'-O-(4,4''-dimethoxytrityl)thymidine, was used as a support and the 5'-dimethoxytrityl group was cleaved off with trichloroacetic acid before synthesis began. Two thymidine residues were then added, using the 9-fluorenylmethoxycarbonyl monomer(**3**) and a synthesis cycle in which the 5'-protection was removed with 10% DBN solution in dichloromethane (Table 4). The fourth residue was added by means of a conventional 5'-(4,4''-dimethoxytrityl)thymidine-3'-phosphate monomer, thus allowing the resin loading to be easily assayed colourimetrically. A control synthesis (**b**) of $(T)_4$ using 4,4'-dimethoxytrityl monomers throughout was also performed ( Table 5 ). The purity of the two products was compared by HPLC (Fig. 28 and 29 ) and found to be similar but the yield of the 9-fluorenylmethoxycarbonyl synthesis was obviously poorer.

Before cleavage and deprotection, samples of resin from each preparation were assayed for dimethoxytrityl content by treatment with 60% hydrochloric acid in ethanol and measuring the dimethoxytrityl cation absorbance spectrophotometrically at 498 nm. The amount of dimethoxytrityl protected oligonucleotide product was thus measured. For the standard synthesis this



Oligonucleotide (**a**) $T_4$

( Figure 28. )




Oligonucleotide (b) $T_4$ standard synthesis

( Figure 29. )

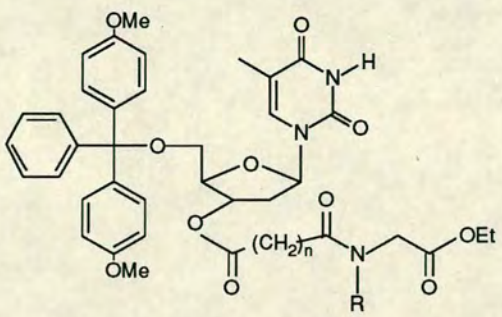
amounted to 37 micromoles oligonucleotide/g resin while for the FMOC prepared sample the resin loading was only 25 micromol/g.

Loss of the oligonucleotide from the resin as a result of succinyl ester linker cleavage was suspected to be the cause of the reduced yield and by soaking the resin in DBN solution it was confirmed that the linker was not stable to strong non-nucleophilic bases ( Table 6).

| Resin | Conditions/Treatment | Half life/minutes |
|---|---|---|
| LCAA cpg (Beckman) | 10% DBU/dichloromethane | 20 |
| Sarcosyl (**14**) | 10% DBU/dichloromethane | >120 hours |
| Phthaloyl (**16**) | 10% DBU/dichloromethane | 3.5 |

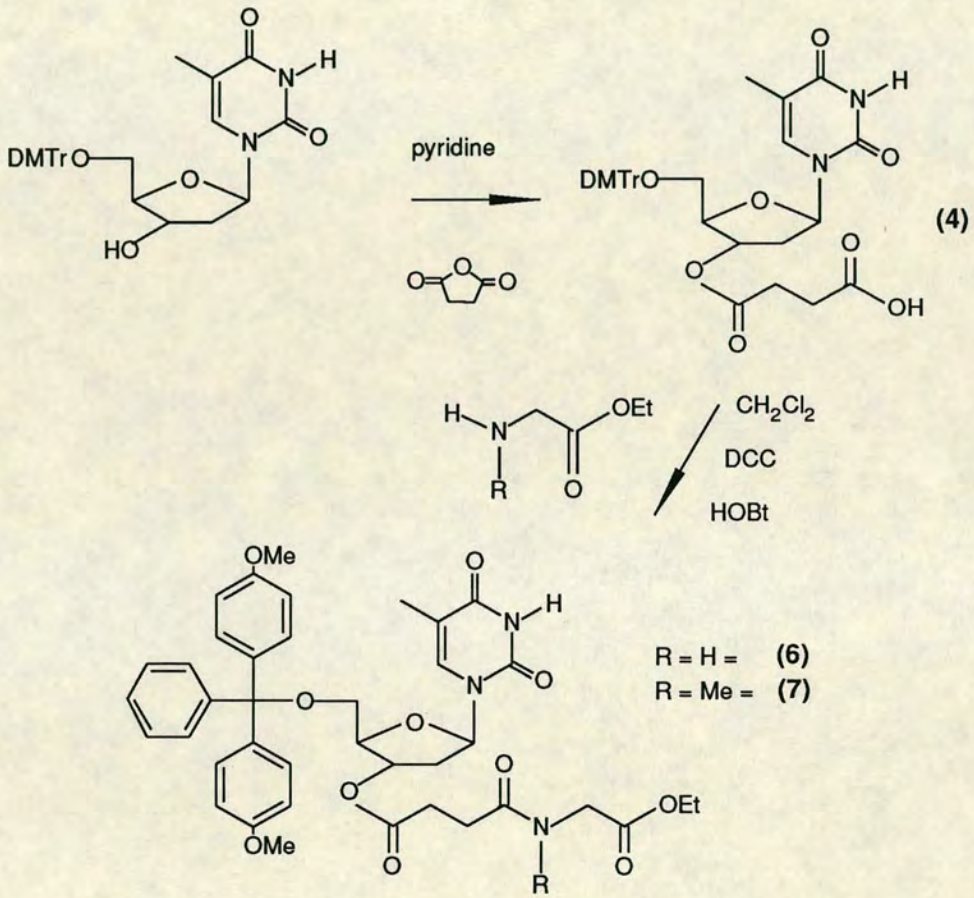
( Table 6. Resin Stability )

Chattopadhyaya and coworkers had also noticed significant instability of the linkage between the oligonucleotide and the support under similar conditions. They reported approximately 1% cleavage every 18 seconds upon treatment with 30 equivalents DBU in dry acetonitrile. This cleavage is thought to occur by a deprotonation mediated elimination mechanism.The strong base (DBU or DBN) removes the proton from the nitrogen of the amide bond joining the alkylamine spacer arm to the succinyl nucleoside and this is followed by a ring closure reaction to give the stable succinimide and the free nucleoside. Thus if the anion at nitrogen was prevented from forming, this reaction could be prevented. In order to test this theory a series of model compounds were prepared (Fig. 30).



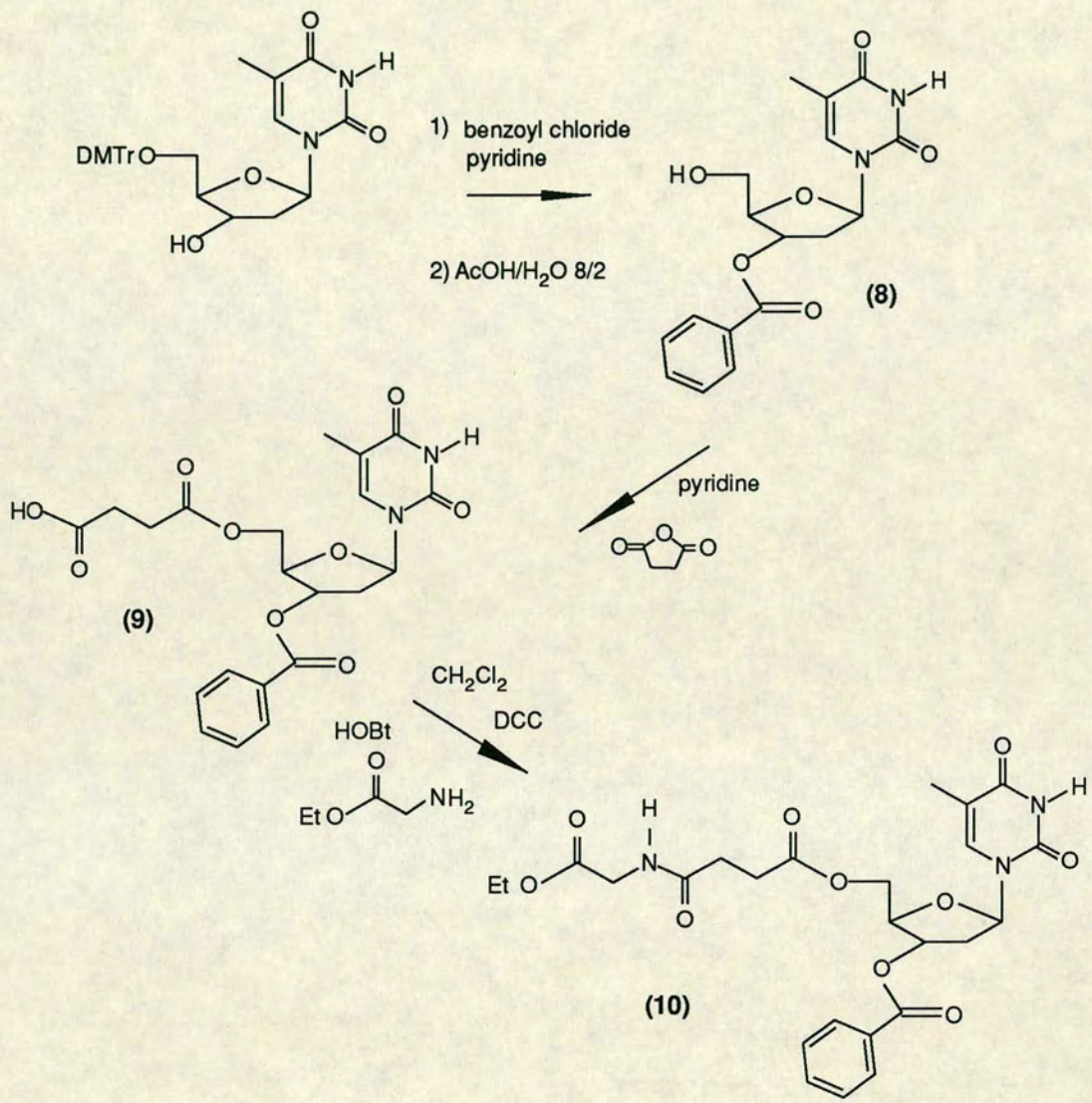

( Figure 30. )

Analogues of the linkage to the resin were prepared by reaction of 5'-O-(4,4''-dimethoxytrityl)-thymidine-3'-O-succinate (**4**) with glycine and sarcosine ethyl esters(Scheme 16). As predicted the glycine model compound(**6**) was completely destroyed by non-nucleophilic bases in under 5 minutes (monitoring by t.l.c.) whereas the sarcosyl model (**7**), incapable of forming an anion, was stable to the same conditions for more than 48 hours.




( Scheme 16. )

The succinyl glycine ester compound may be of use as a protecting group in its own right. A 5'-O-(glycylsuccinyl)-3'-O-benzoylthymidine(**10**) was prepared as a crystalline compound (Scheme 17) and deprotected with DBU to give the free 5'-hydroxyl compound in tlc monitored tests.The linkage appears to be slightly more stable than 9-fluorenylmethoxycarbonyl, being intact after treatment with triethylamine for 1 hour. Attempts to introduce the succinylglycyl moiety specifically at the 5'-hydroxyl without protection of the other hydroxyl functions were not successful. This




( Scheme 17. 5'-O-succinyl glycine model compound)

| Compound | Treatment | Half life |
|---|---|---|
| 5'-DMTr-3'-succinyl-glycine ethylester (**6**) | 10% DBU in acetonitrile | instant cleavage |
| 5'-DMTr-3'-succinyl-sarcosylethylester (**7**) | 10% DBU in acetonitrile | >72 hour |
| 5'-DMTr-3'-glutaryl glycine ethylester (**12**) | 10% DBU in chloroform | 2 hours |
| (**12**). " " | 5% piperidine in acetonitrile | 48 hours |

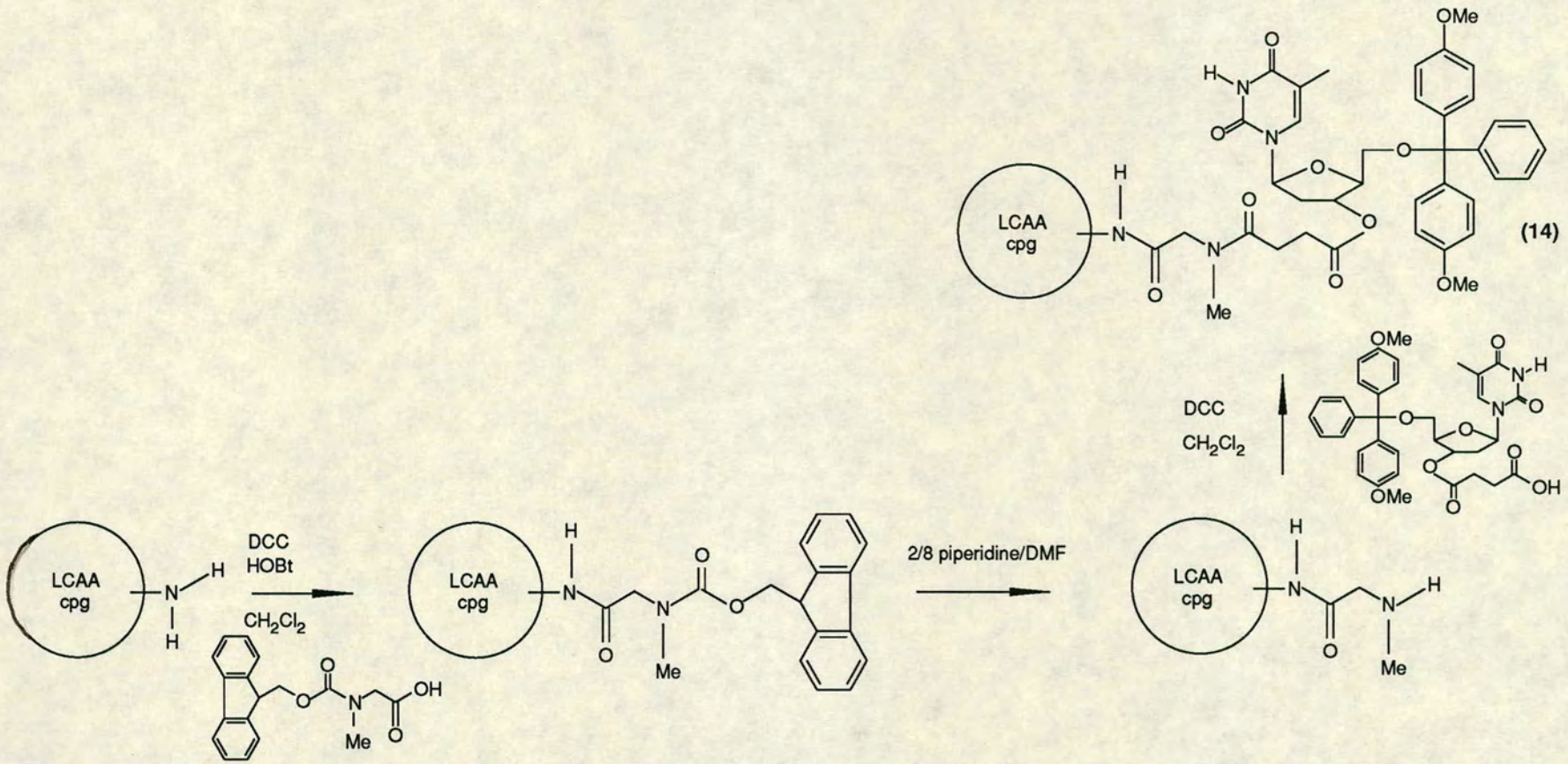
( Table 7. Model compound stability )

group would have the advantage of being produced from readily available starting materials. However it has no uv activity or colour by which to monitor its deprotection. Groups with similar cleavage mechanisms that do contain reporter groups could in principle be designed.

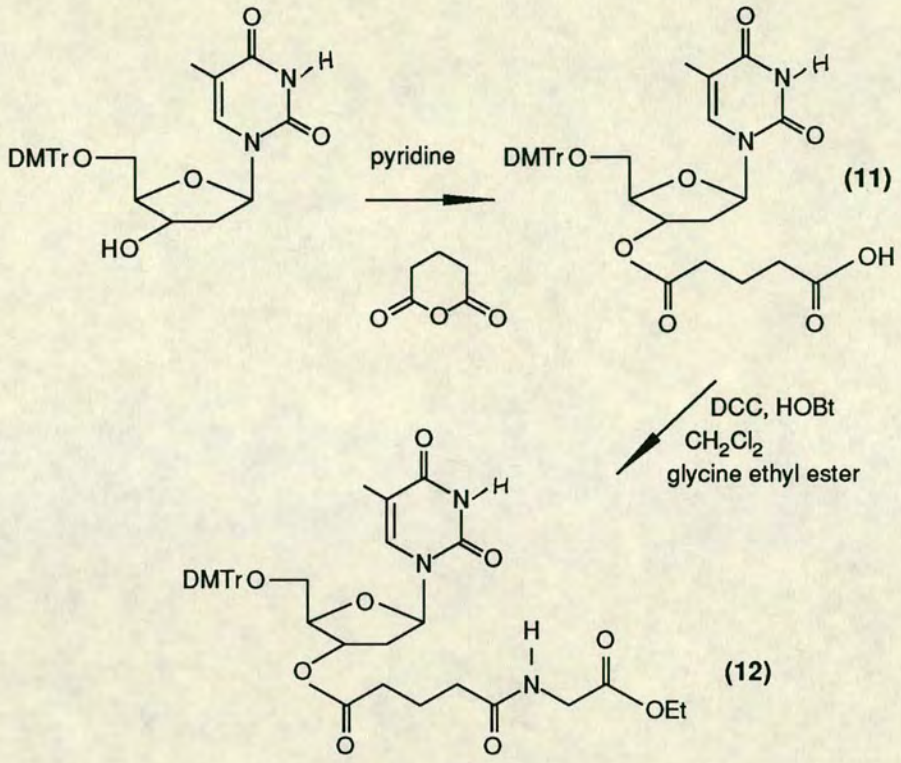
A resin was prepared with a sarcosyl spacer between the alkylamino chain and the 3'-succinate of the first nucleoside(compound (**14**), Scheme 18). This was found to be stable to DBU in acetonitrile or dichloromethane, being cleaved by 10% DBU in acetonitrile at a rate of 0.4% per hour (Table 6). This small amount of cleavage may be due to moisture.

If the proposed mechanism of non-nucleophilic base cleavage of the model compounds and resin is correct, then increasing the size of the product ring from 5-membered to 6-membered would be expected to slow down the rate of cleavage, supposing this ring closing step to be rate limiting[107]. A further model compound was therefore synthesised by condensing a thymidine 3'-O-glutarate (**11**) with glycine ethyl ester (Scheme 19). This compound (**12**) proved to be more stable to DBU solutions than the succinyl glycyl model, having half life of approximately 1 hour (Table 7).

Conversely when a 3'-O-phthaloyl resin (**16**) without sarcosyl spacer group was prepared it was found to be extremely labile ($t_{1/2}$ < 5minutes) as opposed to the succinyl resin $t_{1/2}$ of 30 minutes (Table 6). The phthaloyl linker has an ideal rigid shape for the ring closure reaction.




( Scheme 18. Preparation of Sarcosyl Resin )




( Scheme 19. )

## 2.2 **Applications of Sarcosyl Resin**

The sarcosyl resin was initially tested with a routine 5'-O-(4,4''-dimethoxytrityl)-3'-O-(2-cyanoethoxy)-N,N-diisopropylphosphoramidite synthesis of d(GAA GAA CTG GTA GAG TCG GT) (**c**) using synthesis cycle SSCE103A (Appendix A), (Table 8 ). Coupling efficiency, monitored by dimethoxytrityl analysis, averaged at 98% and the overall yield was calculated to be 65%. The product was found to have equal purity by HPLC ( Fig. 31 ) to a product of the same sequence synthesis (**d**) prepared on commercial succinyl controlled pore glass.

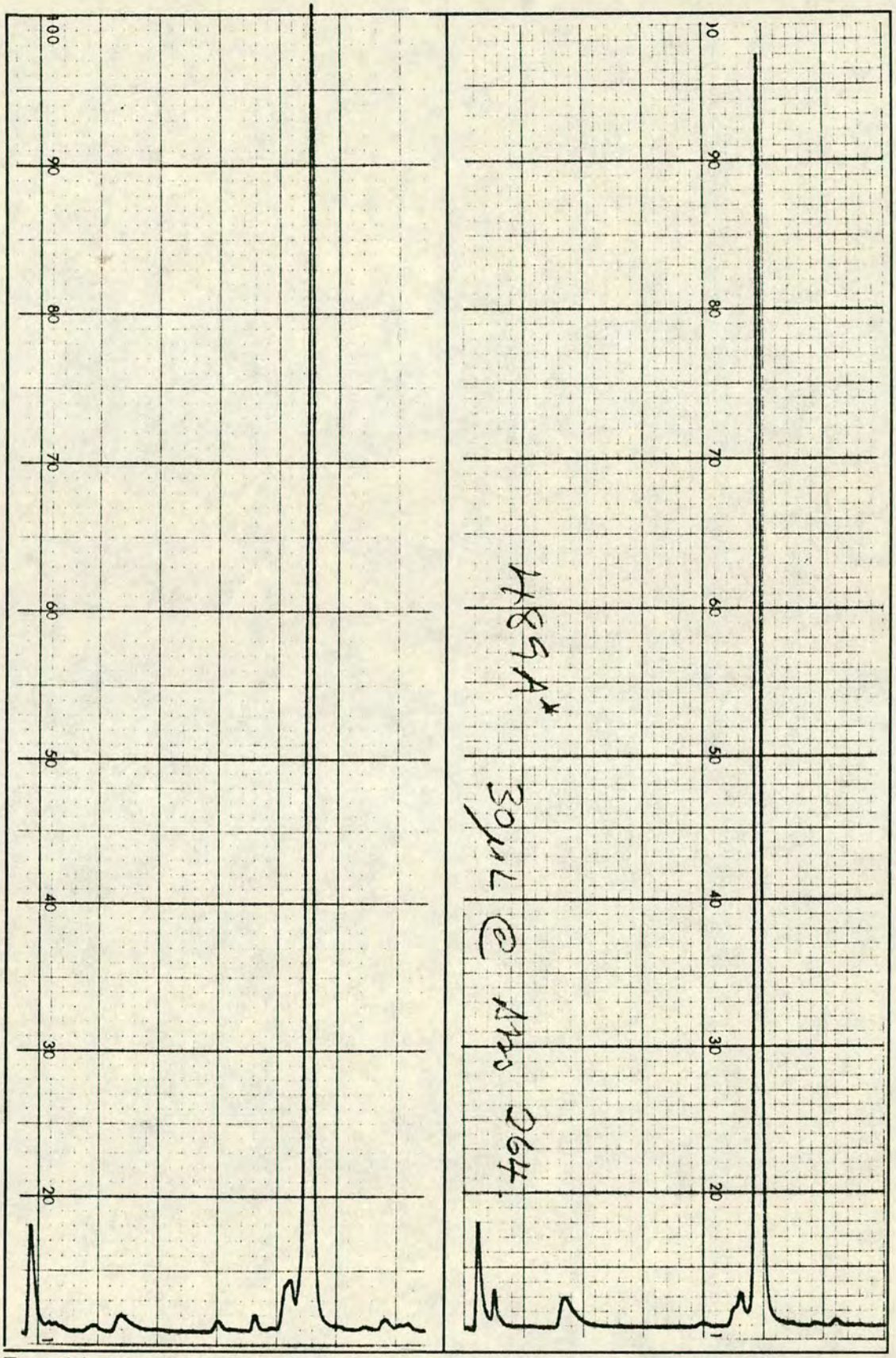
A 5'-O-(9-fluorenylmethoxycarbonyl)-6-N-benzoyl-2'deoxyadenosine-3'-O-methoxy-N,N-diisopropylphosphoramidite(**20**) was prepared (scheme 20) and the deoxyoligonucleotide $d(A)_8$ synthesis(**f**) was synthesised .The resin was initially deblocked with 3% trichloroacetic acid in dichloromethane and then cycle SSME103A(Appendix A) (Table 9 ) was used with 10% DBU in acetonitrile for 5'-deprotection of the 9-fluorenylmethoxycarbonyl group and capping with 10% trimethylsilyl chloride in pyridine[66]. Methyl protection of the phosphotriester was removed by

| Operation | Reagent/solvent | Time/s |
|---|---|---|
| 1. Wash | dichloromethane | 30 |
| 2.deblock 5' | 3% trichloroacetic acid in dichloromethane | 50 |
| 3. wash | dichloromethane | 30 |
| 4.wash | anhydrous acetonitrile | 60 |
| 5.coupling step | 0.1M phosphoramidite in acetonitrile<br>0.5M tetrazole in acetonitrile | 30 |
| 6.capping step | acetic anhydride/lutidine/THF 1/1/8<br>17.6% N-methylimidazole in THF | 40 |
| 7.oxidation | 0.1M $I_2$/water/pyridine/THF<br>2/20/80 | 50 |

( Table 8. SSCE103A )

| Operation | Reagent/solvent | Time/s |
|---|---|---|
| Wash | dichloromethane | 30 |
| deblock 5' | 10% DBU in acetonitrile | 50 |
| wash | dichloromethane | 30 |
| wash | anhydrous acetonitrile | 60 |
| coupling step | 0.1M phosphoramidite in acetonitrile<br>0.5M tetrazole in acetonitrile | 30 |
| capping step | 10% trimethylsilyl chloride in<br>pyridine | 40 |
| oxidation | 0.1M $I_2$/water/pyridine/THF<br>2/20/80 | 50 |

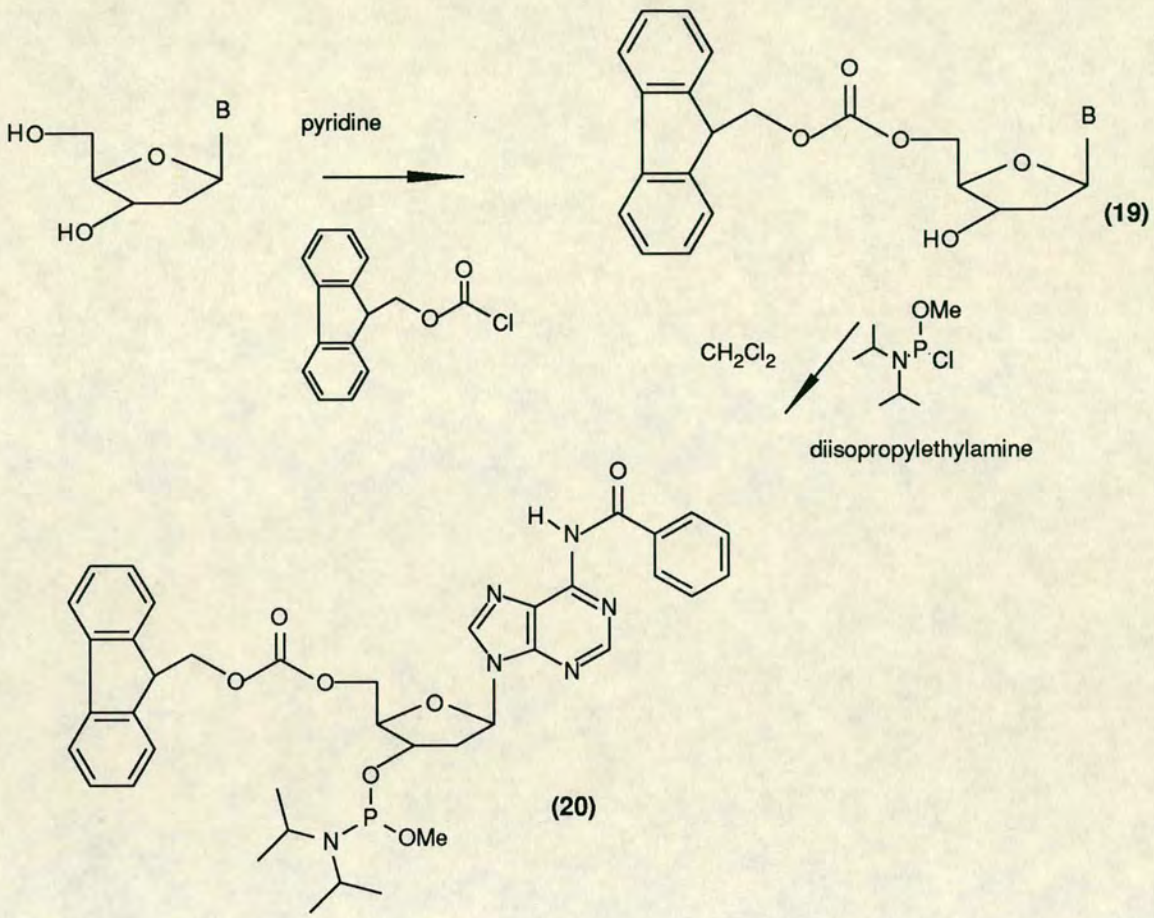
( Table 9. )




Test 1(**c**), 0.03ml/1ml at 2.56AUFS, 264nm, Ion Exchange HPLC

489A (**d**), 0.03ml/1ml at 2.56AUFS 264nm, Ion Exchange HPLC

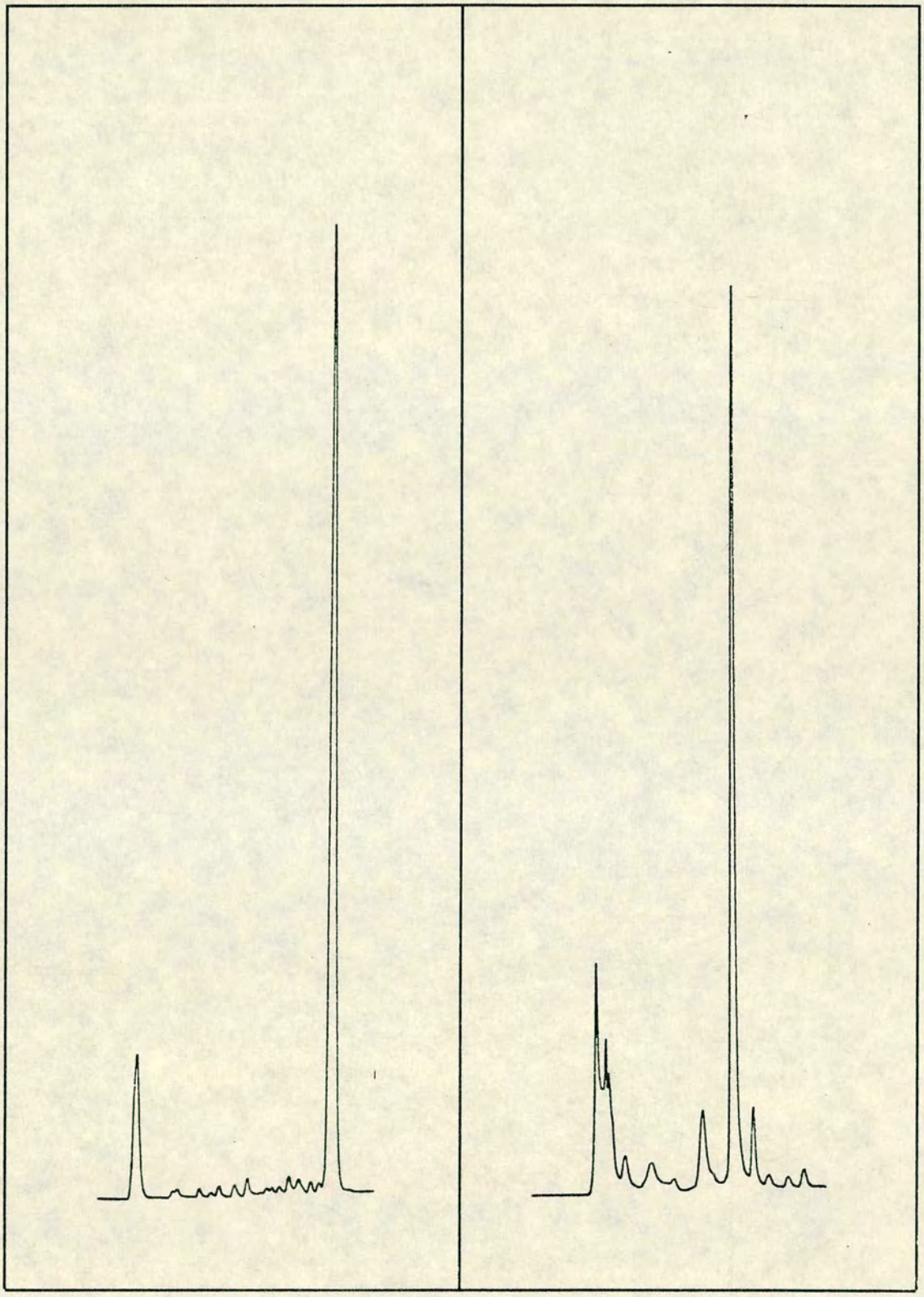
( Figure 31. )




( Scheme 20. )

treatment with thiophenol and triethylamine and the oligonucleotide cleaved from the support with concentrated ammonia solution (Appendix A, end procedure depr ) (Table 12 ). Acyl base protection was removed by treatment with concentrated ammonia solution at 55°C for 6 hours and the product analysed by HPLC ( Fig. 32 ) comparison with a standard $d(A)_8$ product from a synthesis (**e**) using 4,4'-dimethoxytrityl,cyanoethylphosphoramidite monomers (Fig. 33). The test product showed reasonable purity but was obtained in lower yield than the standard. This is found to be the case for all 9-fluorenylmethoxycarbonyl prepared oligonucleotides.

The trimethylsilyl chloride capping reagent was abandoned in later syntheses because of the risk of creating insoluble salts and damaging the synthesiser. Capping in subsequent syntheses was performed with acetic anhydride and lutidine in THF, with N-methylimidazole catalysis synthesis (**g**) (Fig. 35).



d $(A)_8$ (**f**), 0.05ml/1ml at 0.64AUFS

Monitored at 254nm

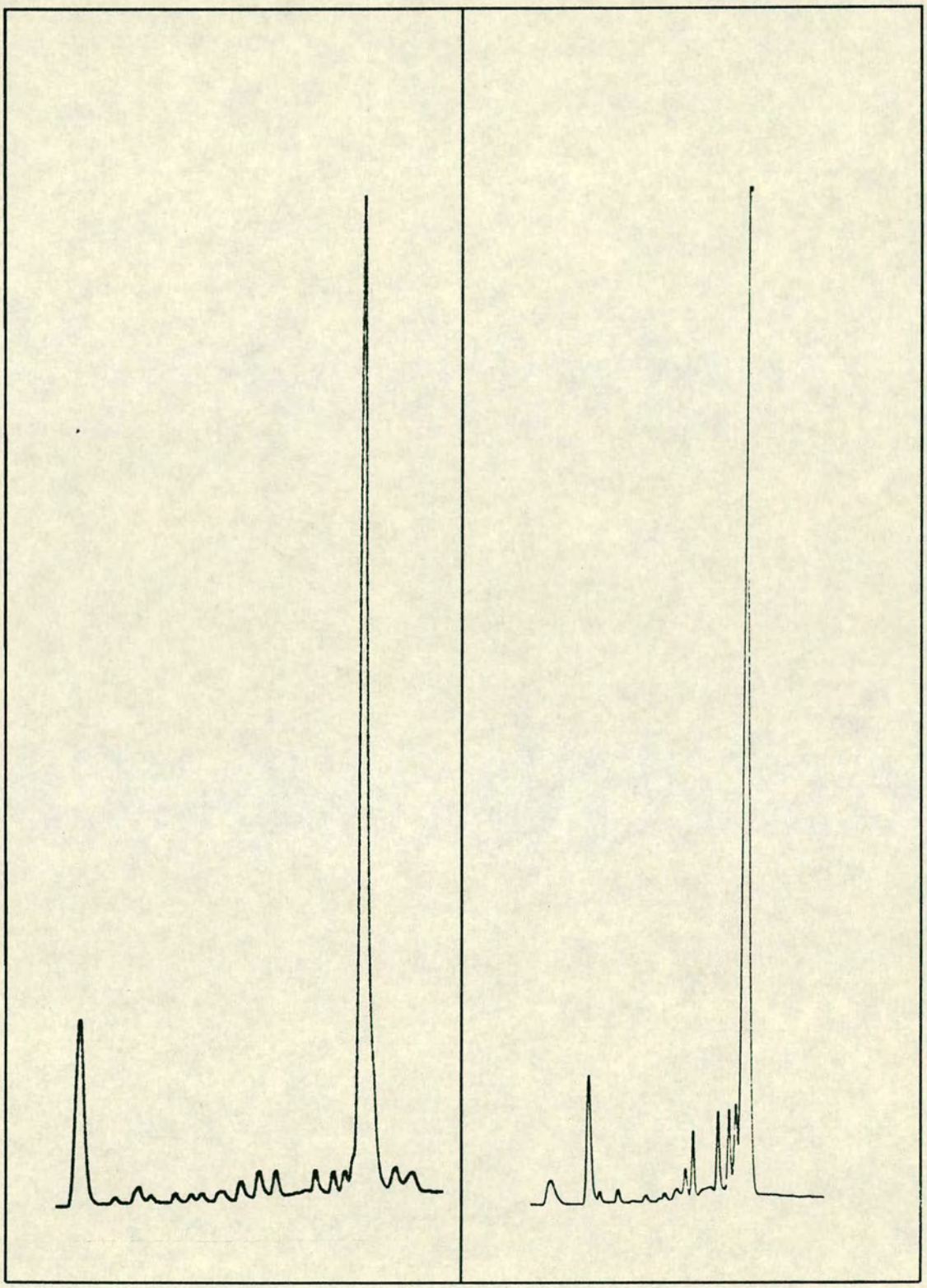
Ion Exchange HPLC

d $(A)_8$ (**e**), 0.015ml/1ml at 0.64AUFS

Monitored at 254nm

Ion Exchange HPLC

( Figure 32. )



d $(A)_8$ (**f**), 0.05ml/1ml at 1.28AUFS

Monitored at 254nm

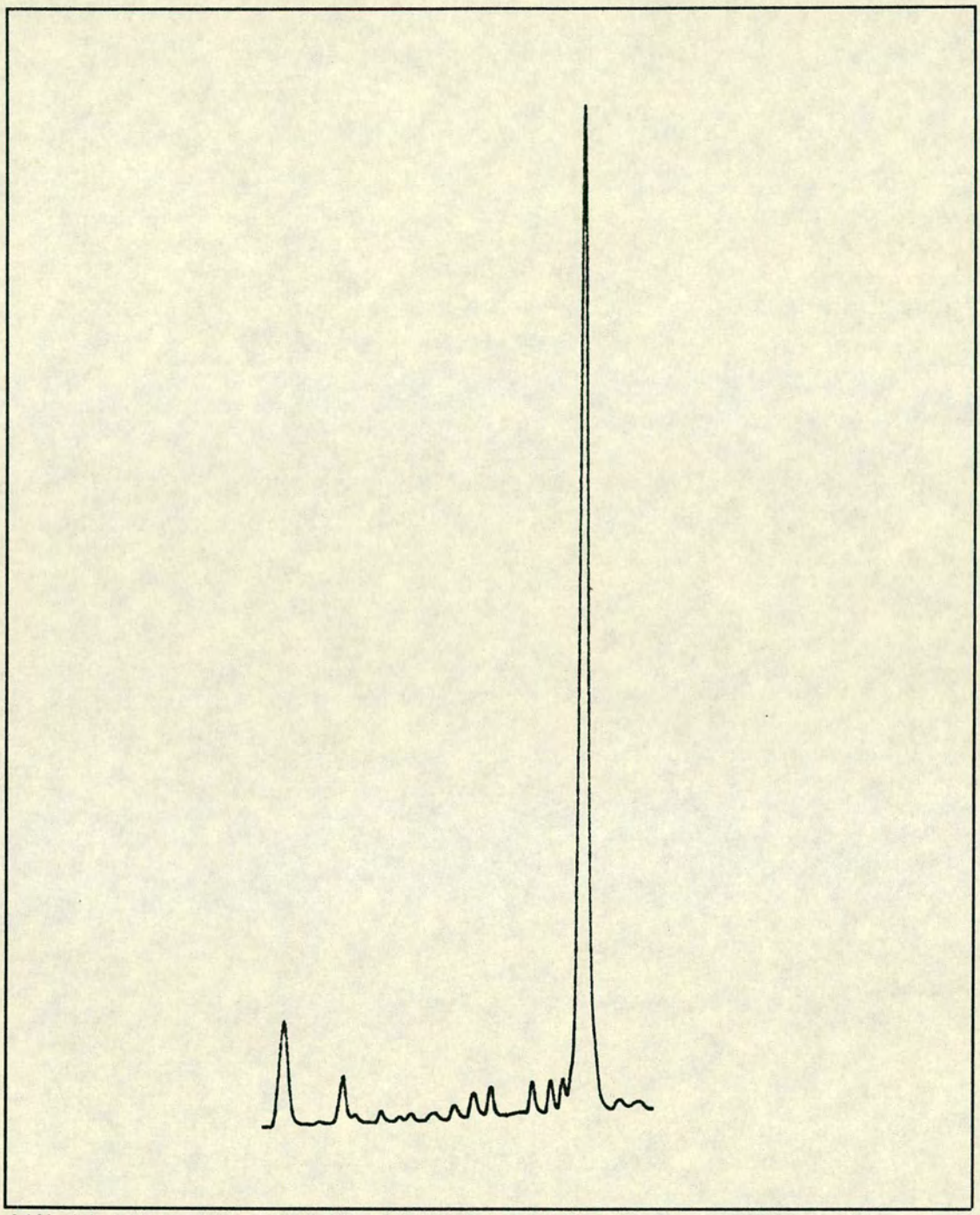
Reversed Phase HPLC

d $(A)_8$ (**e**), 0.05ml/1ml at 1.28AUFS

Monitored at 254nm

Reversed Phase HPLC(75% actual size)

( Figure 33. )

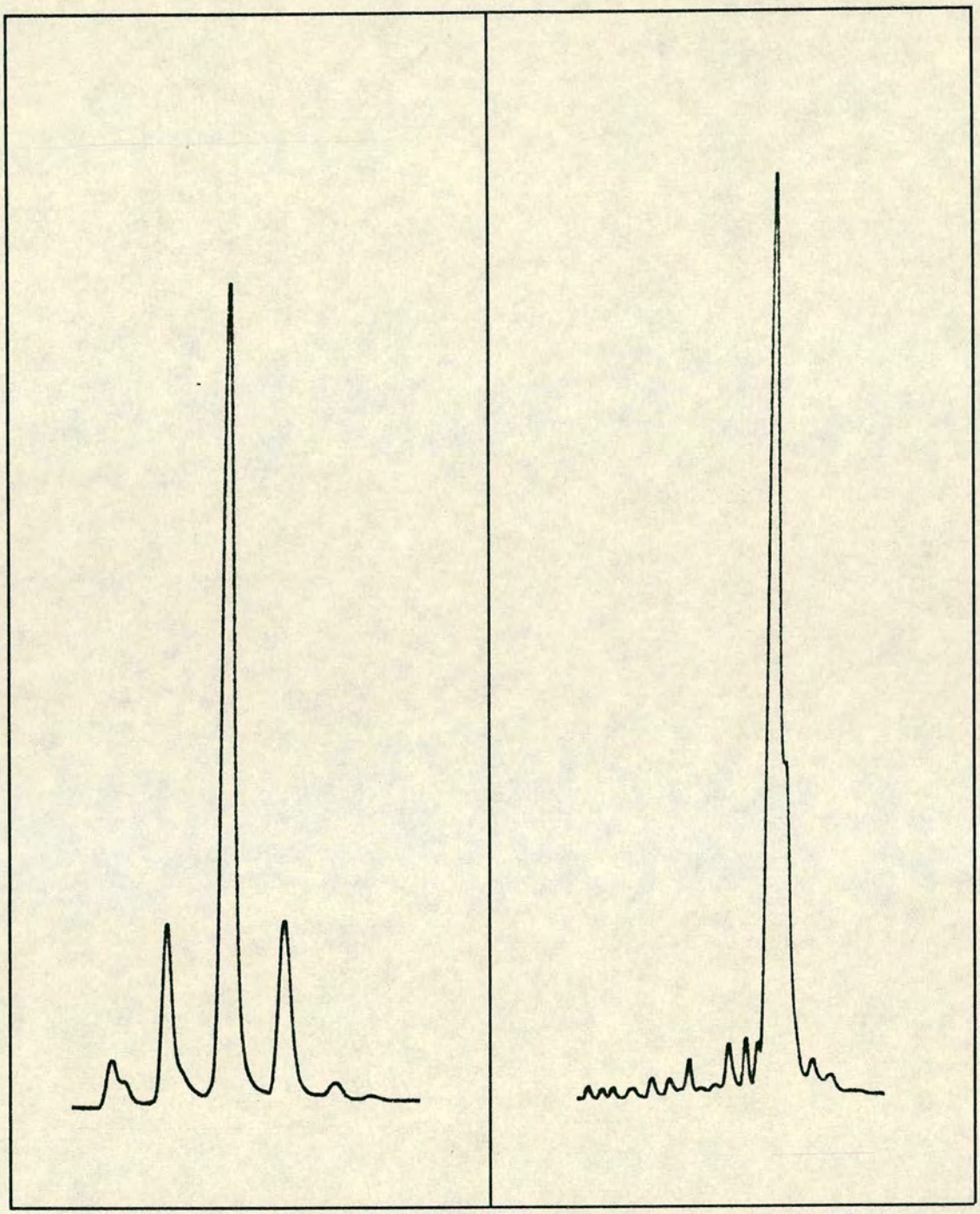


d $(A)_8$ (**e**), 0.025ml/1ml + d $(A)_8$ (**f**), 0.005ml/1ml at 0.64AUFS

Monitored at 254nm

Reversed phase HPLC

( Figure 34. )



d $(A)_8$ (**g**), 0.05ml/1ml at 1.28AUFS

Monitored at 254nm

Ion Exchange HPLC

d $(A)_8$ (**f**), 0.025ml/1ml at 2.56AUFS

Monitored at 254nm

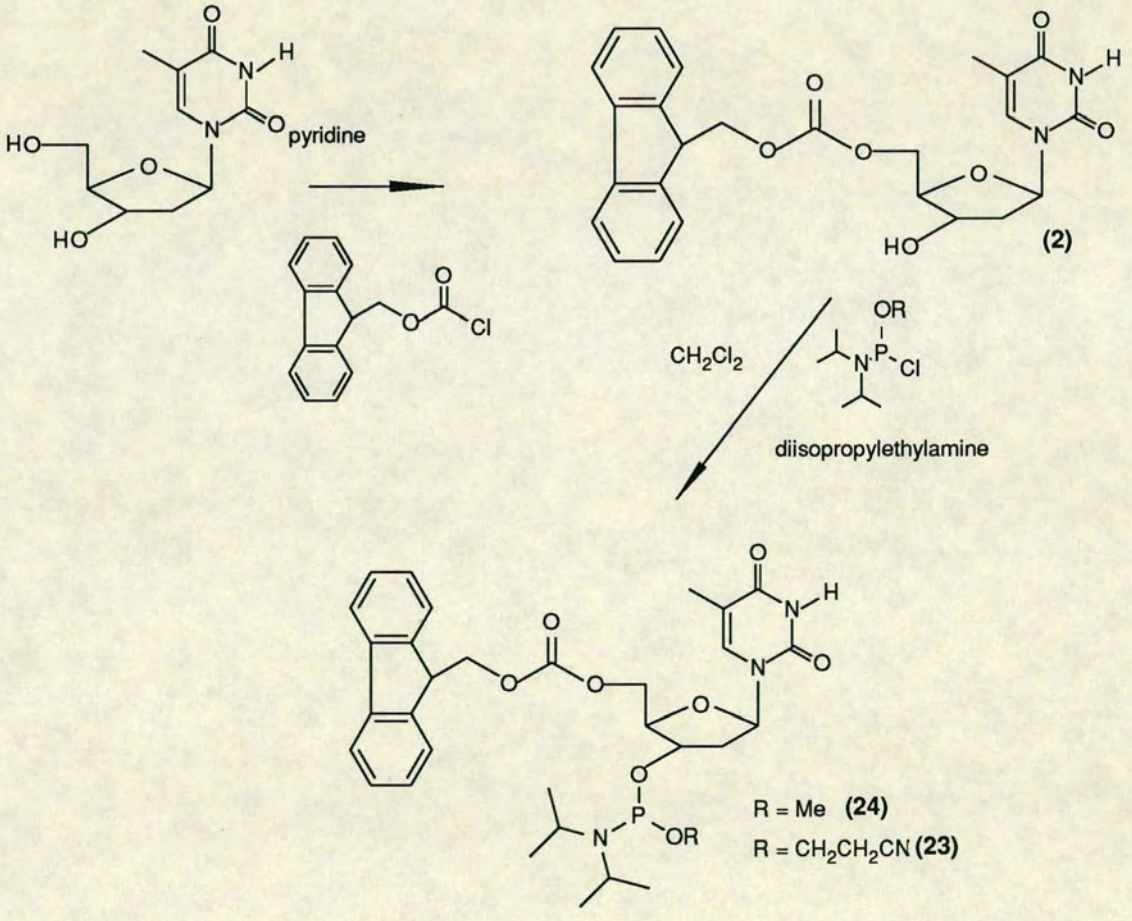
Ion Exchange HPLC

( Figure 35. )

A 9-fluorenylmethoxycarbonyl protected resin was not produced because of the difficulty of synthesising a 5-O-(9-fluorenylmethoxycarbonyl)-thymidine-3'-succinate and of assessing the loading of the resulting resin.

## 2.3 **The Synthesis of Hexathymidylic acid: A Comparison of Five Synthetic Strategies.**

5'-O-(9-Fluorenylmethoxycarbonyl)-thymidine-3'-O-N,N-diisopropylmethoxyphosphoramidite (**23**) and 5'-O-(9-fluorenylmethoxycarbonyl)-thymidine-3'-O-N,N-diisopropylcyanoethylphosphoramidite (**24**) were synthesised (Scheme 21) and the deoxyoligonucleotide d $(T)_6$ was selected as a test sequence to compare a variety of synthetic schemes (Table 10 ) (Fig. 36 - 42).




( Scheme 21. )

| $_{-}T_6$ | 5'-protection | 5'-cleavage | N,N-diisopropyl phosphoramidite | resin type |
|---|---|---|---|---|
| **h** | FMOC | piperidine | methyl | sarcosyl |
| **i** | FMOC | piperidine | methyl | Beckman(succinyl) |
| **j** | DMTr | trichloroacetic acid | cyanoethyl | Beckman(succinyl) |
| **k** | FMOC | piperidine | cyanoethyl | Beckman (succinyl) |
| **l** | FMOC | DBU | cyanoethyl | sarcosyl |

( Table 10. )

Short oligonucleotide sequences have the advantage that base modifications are clearly seen in their reversed phase HPLC . The use of strong non-nucleophilic bases with thymidine and trimethylphosphate in a model study has been found to cause base methylation[108].The thymine base has pKa of 9.94 (25°C) and can therefore be deprotonated by bases such as triethylamine (pKa 10.75 ) and piperidine (pKa 11.23), leaving an active anion to accept a methyl group from a phosphotriester. Triethylamine has been shown to cause base methylation at a very slow rate(13% in 2 days) but DBU, with a significantly higher pKa, causes methylation at a much faster rate(95% in 3 hours). This result was confirmed in work done in this laboratory[109] showing that thymidine would need to be protected in order to be used with DBU or DBN solutions. Previous workers[66,67] on FMOC deoxyoligonucleotide synthesis had not reported any base modification side reactions and so syntheses of $T_6$ using 5% piperidine in acetonitrile[66] as a 5'-hydroxyl deblocking agent were performed (syntheses **h,i,** and **k**). A longer deprotection time was necessary with the piperidine solution and therefore a longer cycle time (Table 11 ). However, it was discovered that the commercial long chain alkyl-amine succinyl resin was stable to this solution so subsequent deoxyoligonucleotide syntheses using piperidine solutions were carried

| Operation | Reagent/solvent | Time/s |
|---|---|---|
| wash | acetonitrile | 30 |
| deblock 5' | 5% piperidine in acetonitrle | 180 |
| wash | acetonitrile | 30 |
| wash | anhydrous acetonitrile | 60 |
| coupling step | 0.1M phosphoramidite in acetonitrile<br>0.5M tetrazole in acetonitrile | 30 |
| capping step | acetic anhydride/lutidine/THF 1/1/8<br>17.6% N-methylimidazole in THF | 40 |
| oxidation | 0.1M $I_2$/water/pyridine/THF<br>2/20/80 | 50 |

( Table 11. )

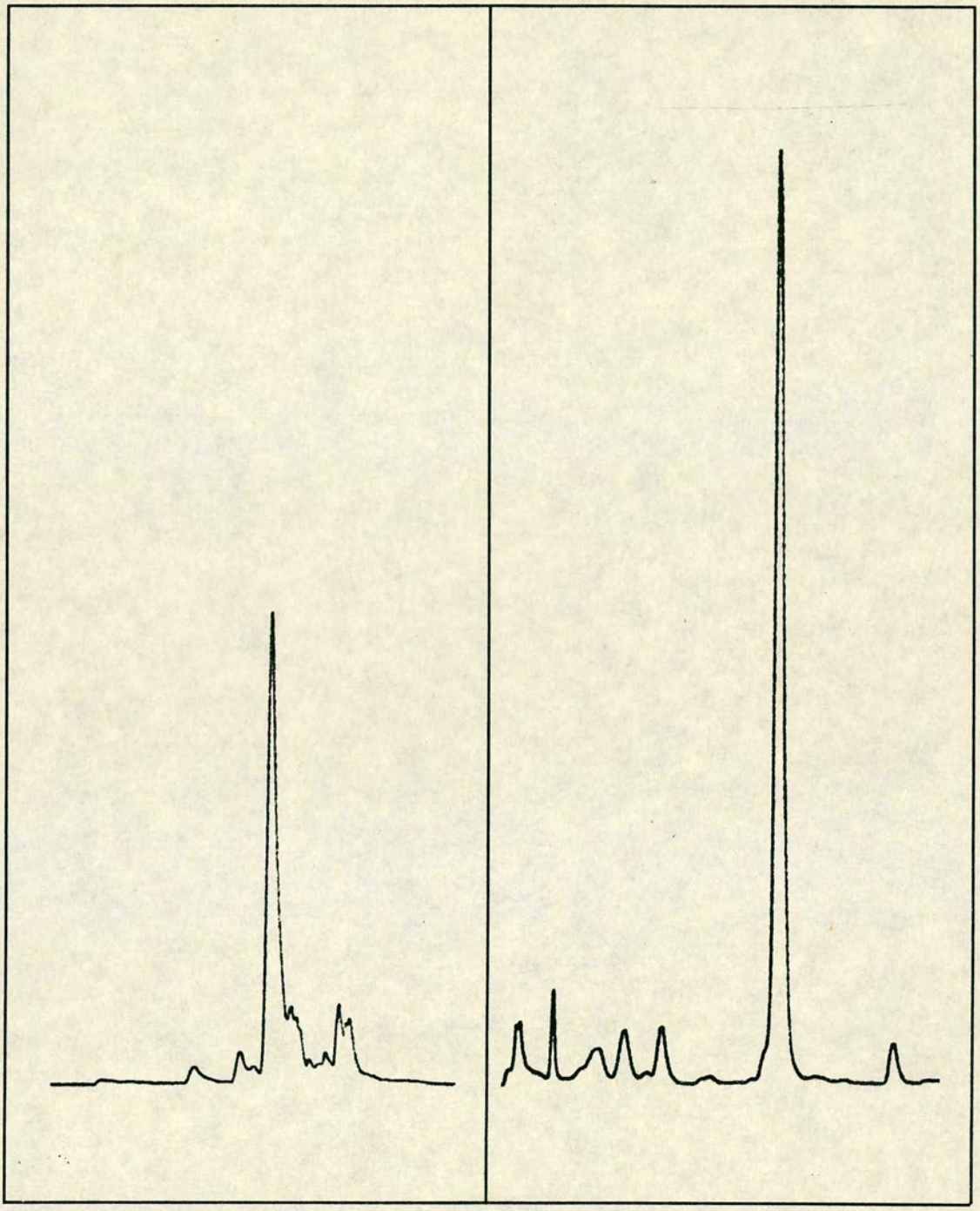
| Operation | Reagent/solvent | Time/mins |
|---|---|---|
| cleave methyl groups | thiophenol/triethylamine/dioxane<br>1/1/2 | 31 |
| wash | methanol | 4 |
| cleave succinyl linker | concentrated ammonia solution | 60 |

( Table 12. )

out with commercial resin (Beckman or ABI.). The 5%piperidine solution in acetonitrile was found not to cause significant base modification( reversed phase HPLC of syntheses ( **h**). and (**i**). as compared with synthesis (**j**). Fig. 36, 37 and 38). Yields from commercial (succinyl ) resin and sarcosyl resin for FMOC syntheses were found to be similar (estimated from HPLC peak integration). Piperidine can thus be used with succinyl linkage, it does not cause base modification side reactions and it is easily dried and purified. It therefore appears to be a useful deblocking reagent in 9-fluorenylmethoxycarbonyl mediated synthesis despite the fact that its use increases the synthesis cycle time.

Of all the syntheses of d $(T)_6$ using 5'-(9-fluorenylmethoxycarbonyl)-protected monomers synthesis (**l**), using sarcosyl resin with 10% DBU solutions and a cyanoethyl phosphoramidite, was the purest by HPLC (Fig. 42) and this scheme would have to be considered if long sequences were attempted. The use of cyanoethyl-phosphoramidites does not lead to alkylation of the thymine base as it is presumed that the cyanoethyl group is removed at every DBU treatment with the beta elimination product of this reaction being acrylonitrile. The growing oligonucleotide in such a synthesis would have a phosphodiester backbone with potential for side reactions. In phosphoramidite synthesis these side reactions are liable to be minimal as, unlike phosphotriester oligonucleotide synthesis, no strongly electrophilic activating agents such as triisopropylbenzenesulphonyl chloride are used. In later work it was decided to employ the milder deprotection conditions and preserve the phosphotriester backbone.

All the syntheses show, in ion exchange HPLC, a small peak which may correspond to a mixture of oligonucleotides of chain length 1 unit longer than the target and it was noted that the monomers had a lifetime of only about 48 hours in acetonitrile (t.l.c. showed lower running spot due to loss of FMOC from the nucleoside and higher running fluorescent spot due to dibenzofulvene). Some of the monomer may thus become 5'-deprotected in acetonitrile solution. Ten equivalents of monomer are added per coupling step and where a free 5'-hydroxyl monomer adds to a growing oligonucleotide it is likely that it will react with a further monomer to give a double addition. The solid 9-fluorenylmethoxycarbonyl monomers do not appear to deteriorate if stored at 4°C.



$T_6$ (**h**), 0.05ml/1ml at 1.0AUFS

Monitored at 264nm

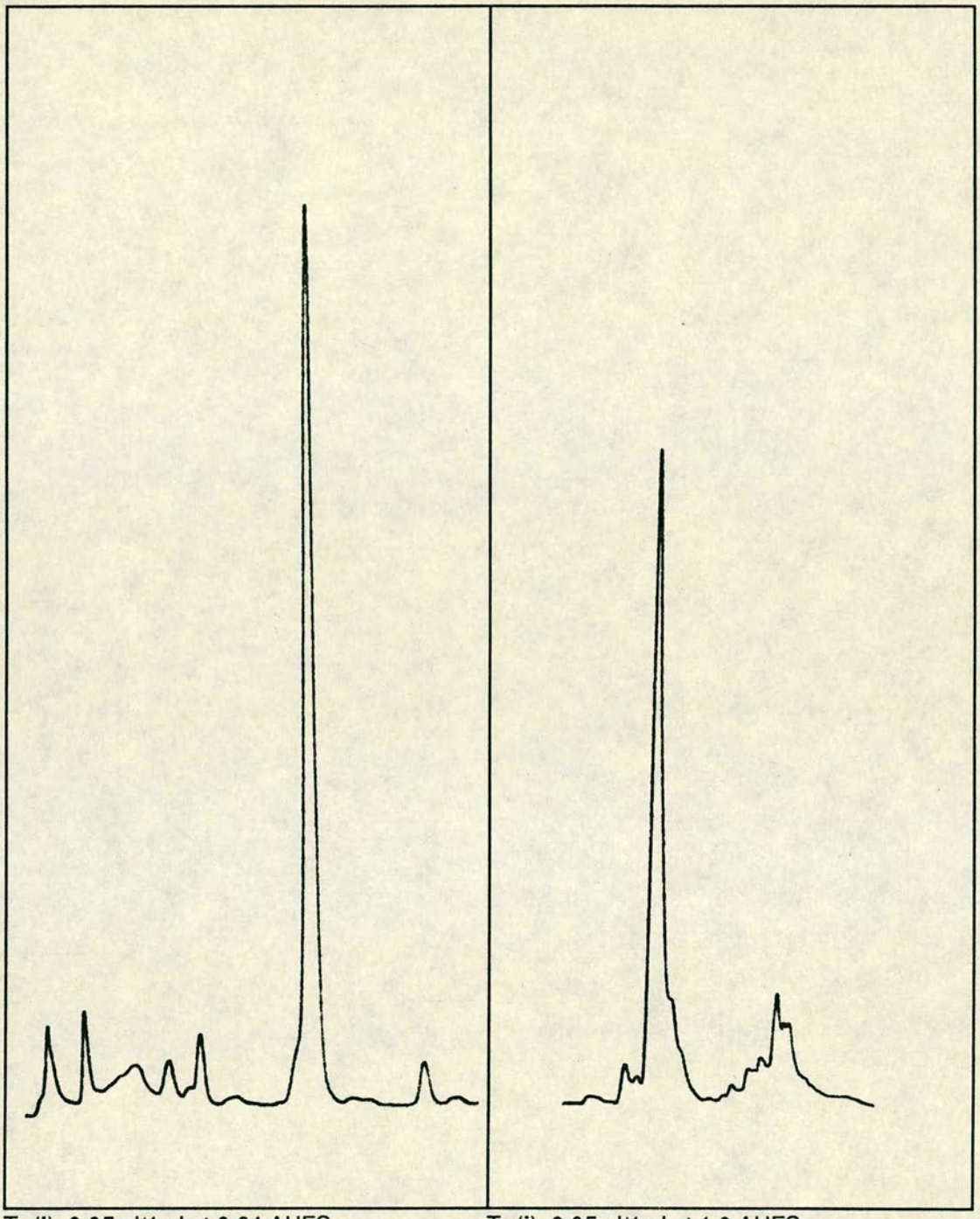
Reversed Phase HPLC

$T_6$ (**h**), 0.05ml/1ml at 0.64AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 36. )



$T_6$ (i), 0.05ml/1ml at 0.64 AUFS

Monitored at 264nm

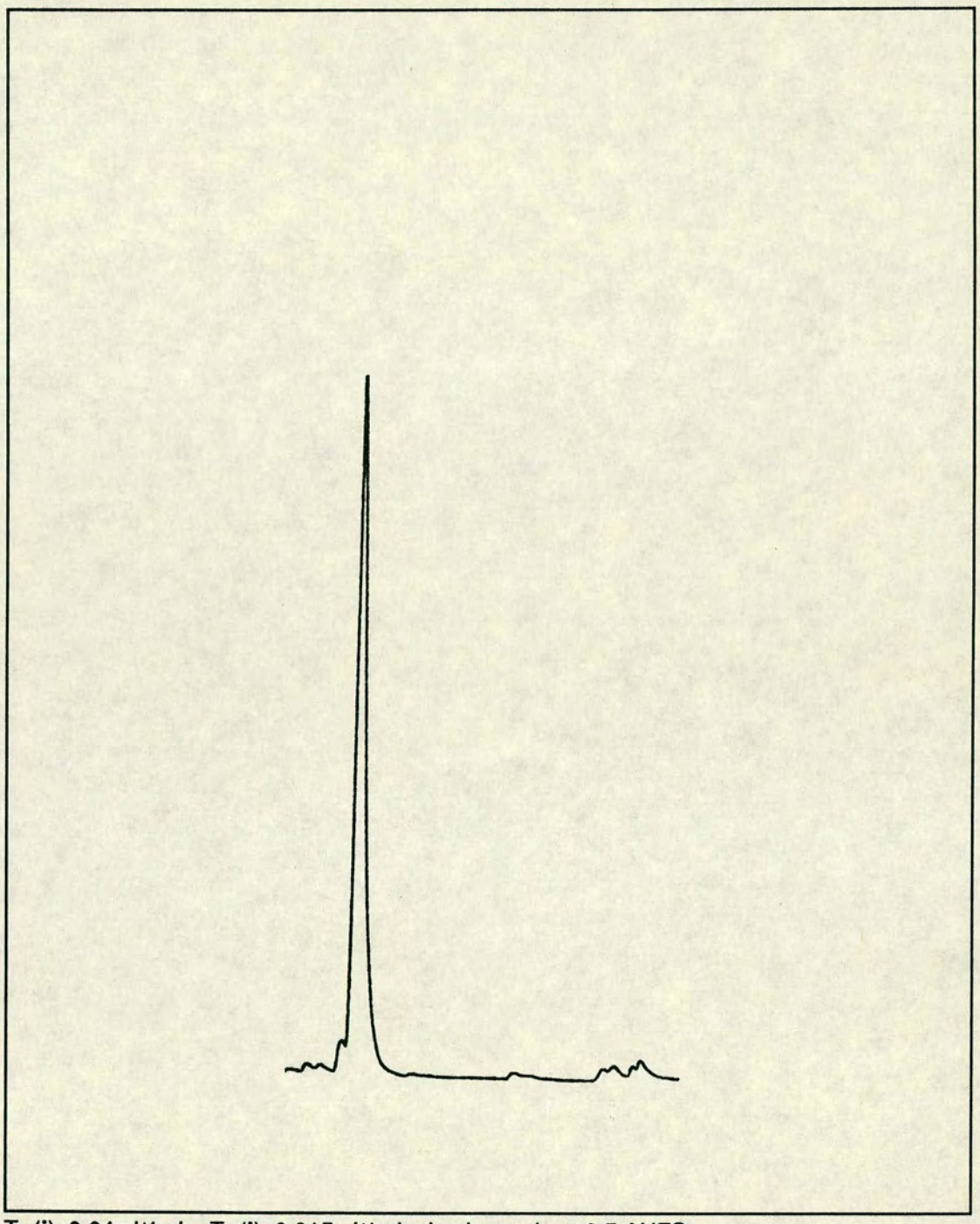
Ion Exchange HPLC

$T_6$ (i), 0.05ml/1ml at 1.0 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 37. )

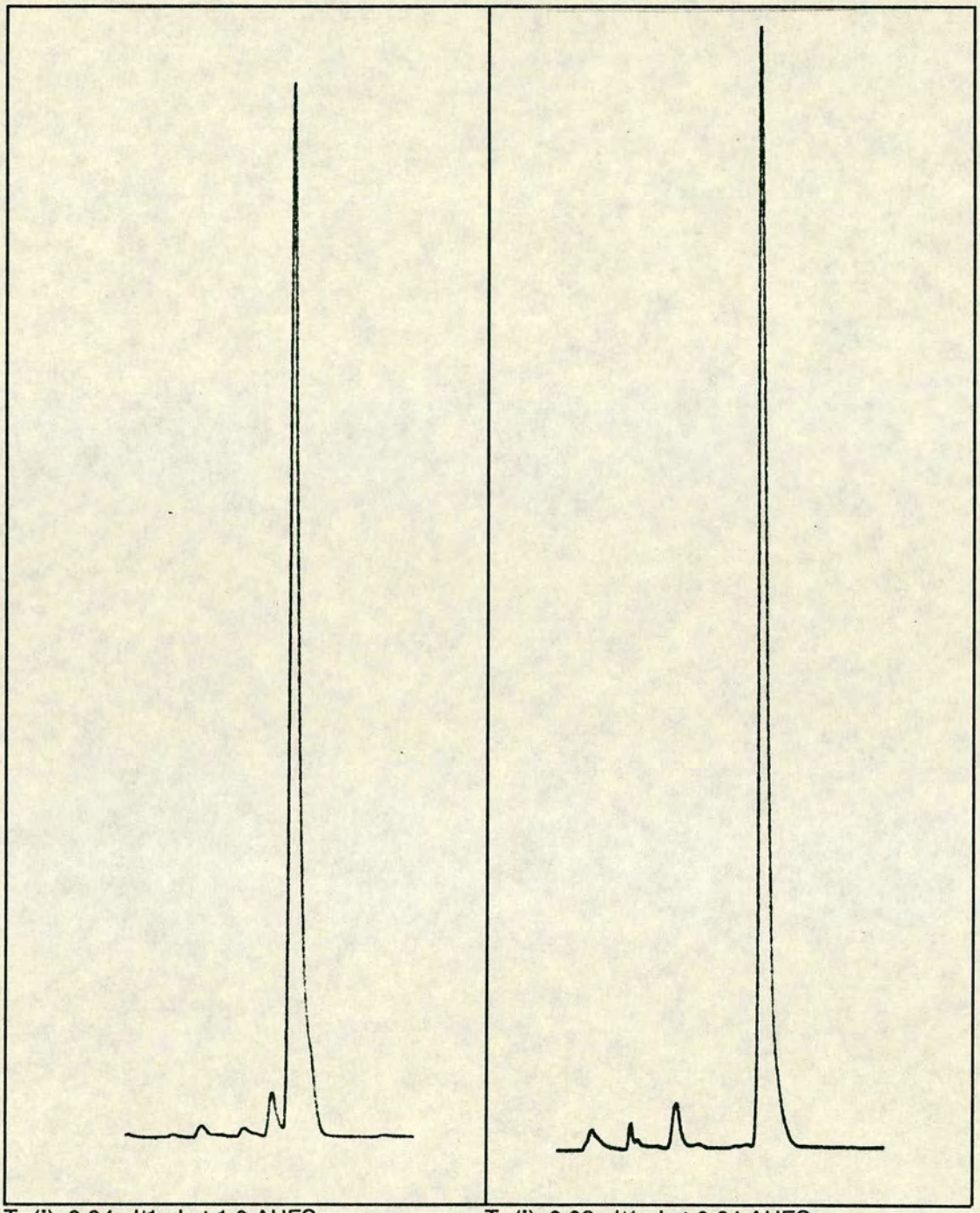


$T_6$ (i), 0.04ml/1ml + $T_6$ (j), 0.015ml/1ml mixed sample at 0.5 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 38. )



$T_6$ (j), 0.04ml/1ml at 1.0 AUFS

Monitored at 264nm

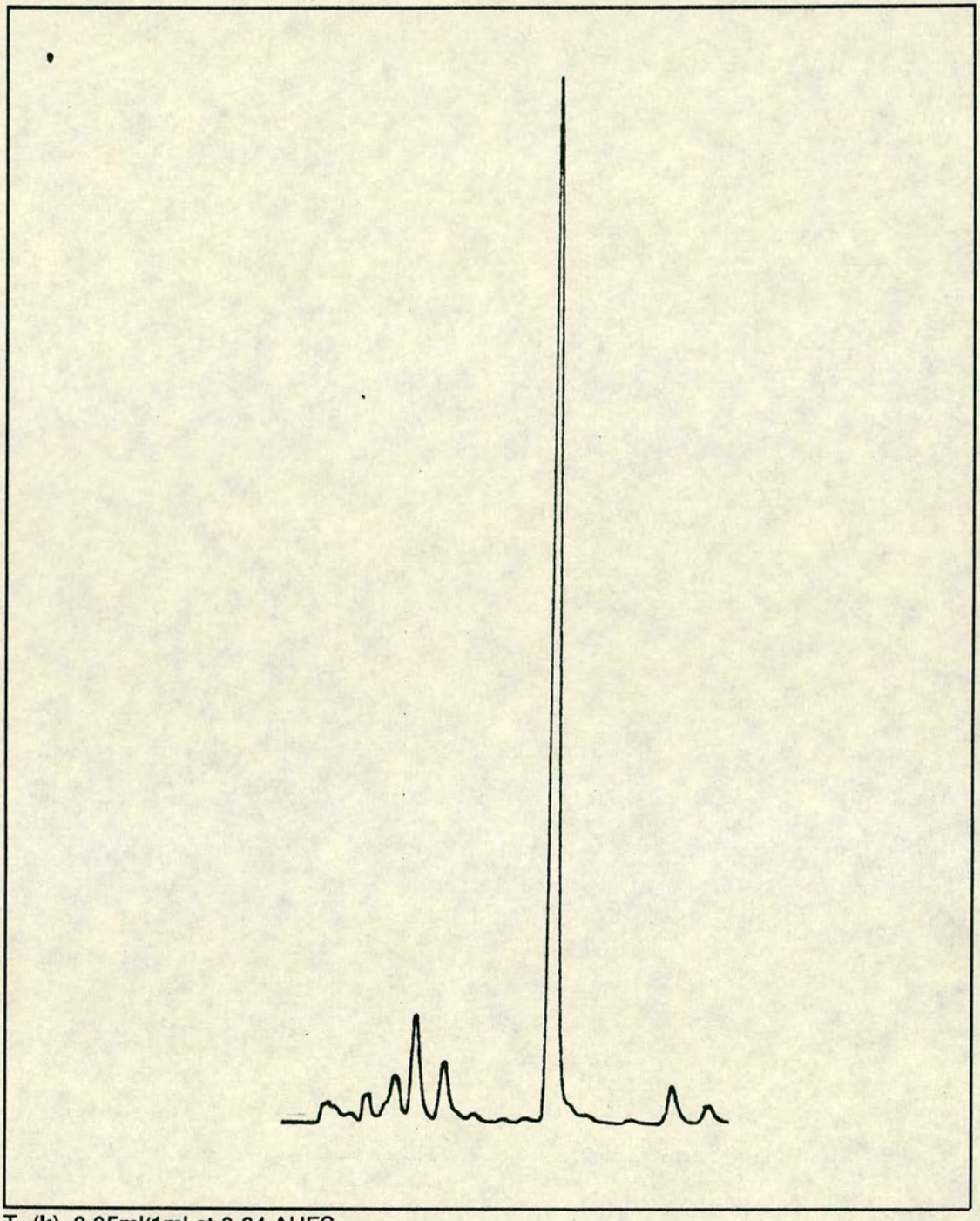
Reversed Phase HPLC

$T_6$ (j), 0.03ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 39. )

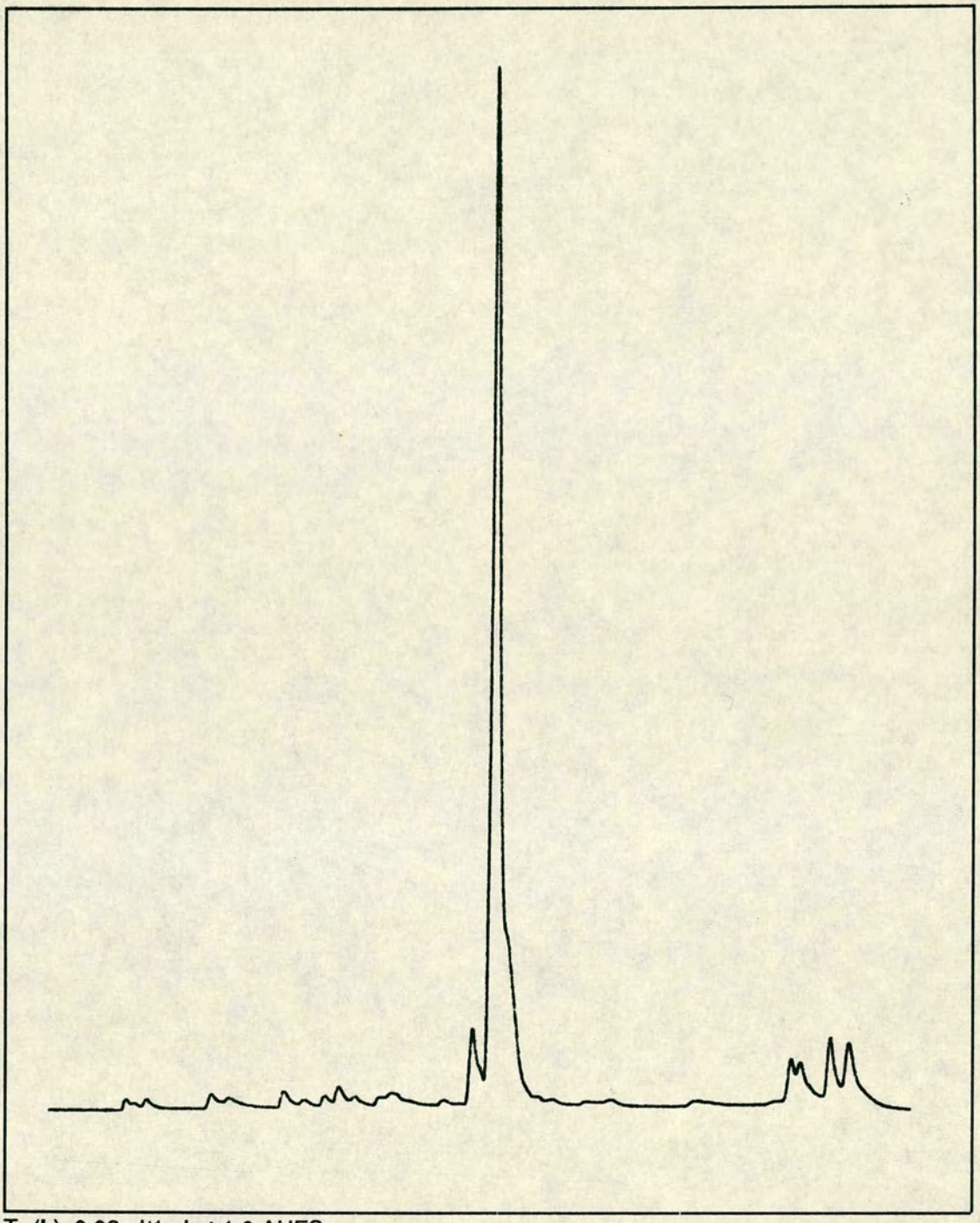


$T_6$ (k), 0.05ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 40. )

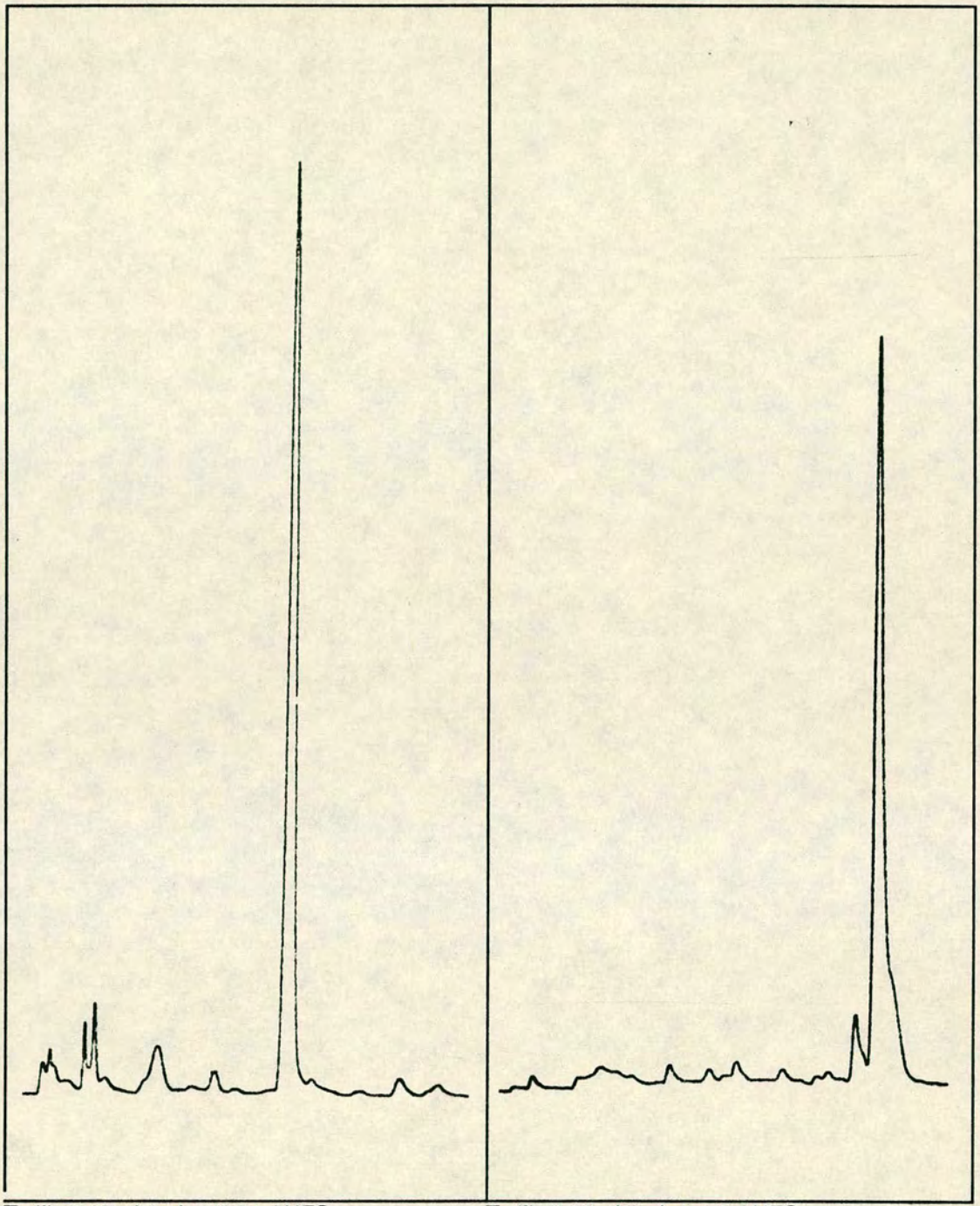


$T_6$ (k), 0.08ml/1ml at 1.0 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 41. )



$T_6$ (I), 0.07ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

$T_6$ (I), 0.08ml/1ml at 1.0 AUFS

Monitored at 264nm

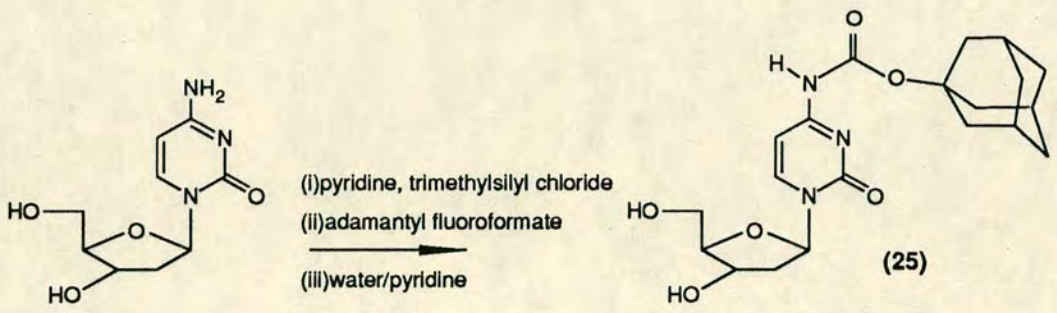
Reversed Phase HPLC

( Figure 42. )

## 2.4 Orthogonal Protection in Oligonucleotide Synthesis

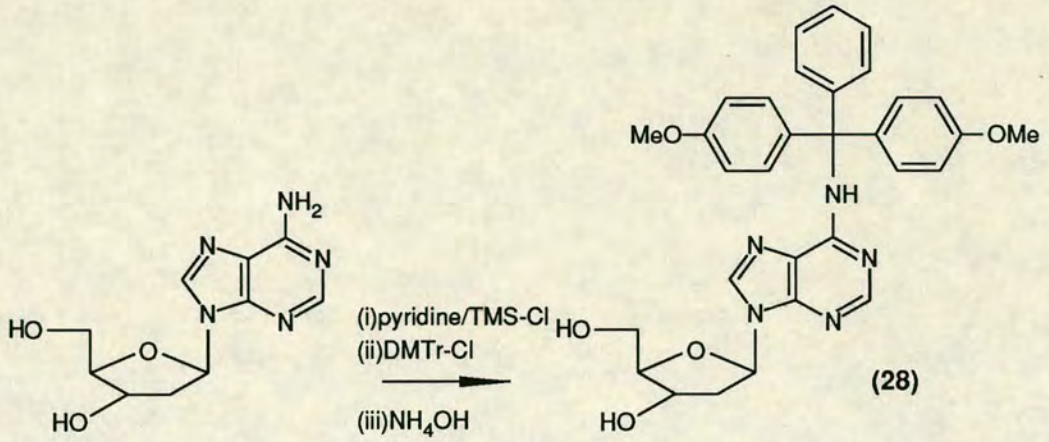
As 9-fluorenylmethoxycarbonyl proved to be effective as a base labile 5'-O-protecting group, acid labile protection could now be applied to the exocyclic amine functions of the bases. This scheme would allow the complete deprotection of an oligonucleotide still bound to the solid support as the linker is stable both to the bases used to remove 9-fluorenylmethoxycarbonyl groups and to acid treatment. The use of such resin bound oligonucleotides for sequence specific hybridisation might then be a possibility. It should be possible to assess the purity of the bound oligonucleotide by cleavage of a sample of the resin, with concentrated ammonia solution, and by subsequent HPLC analysis of the product oligonucleotide.

Initially, adamantyloxycarbonyl protection (**25**) was applied to N(4) of cytidine by transient protection followed by the addition of adamantoylfluoroformate(Scheme 22). However the adamantoyl protection was found to be too stable towards acidolysis for use in oligonucleotide synthesis, where there is the omnipresent risk of depurination.The adamantoyl carbamate was stable to 80% acetic acid but was cleaved quickly by treatment with 60% hydrochloric acid in ethanol.




( Scheme 22. )

4.4'-Dimethoxytrityl groups have been reported for amine protection in oligonucleotide synthesis[114] and so the amino-groups of dC, dG and dA were protected by 4,4'-dimethoxytritylation of trimethylsilyl protected nucleosides(Scheme 23) (**26, 27, 28**) . These compounds could be deprotected instantly by a solution of 3% trichloroacetic acid in dichloromethane. A standard synthesis of $d(A)_{20}$ synthesis(**n**) was carried out to demonstrate that depurination of deoxyadenosine did not seem to occur to a significant extent even after the equivalent of 10 minutes



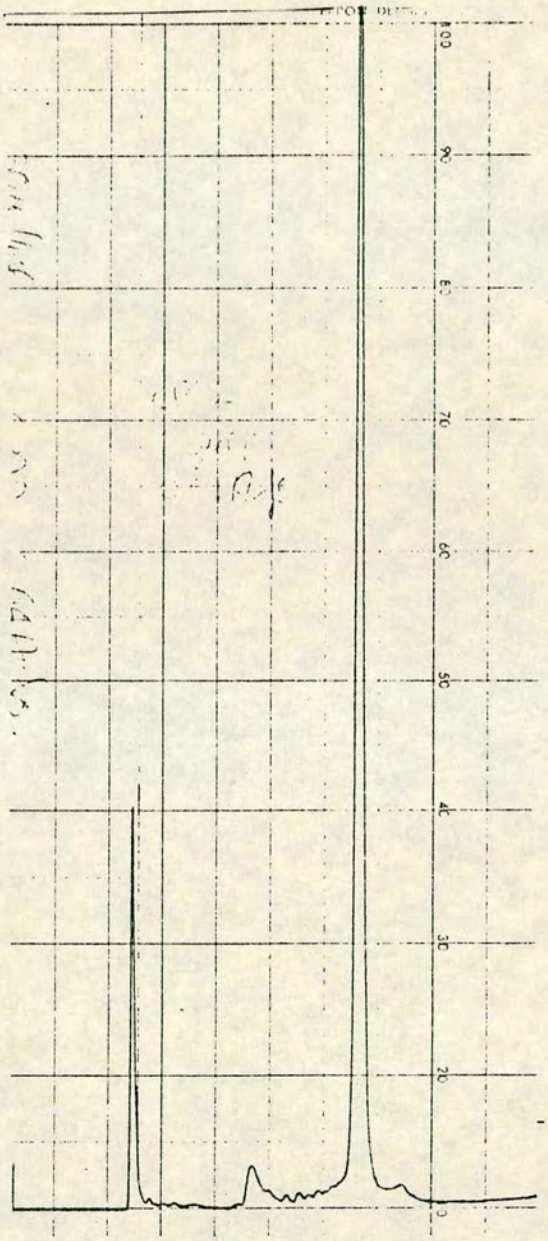

( Scheme 23. )

treatment with trichloroacetic acid (3% solution in dichloromethane)(Fig. 43). The ribose 5'-hydroxyls were protected with 9-fluorenylmethoxycarbonyl groups(**29**, **30** and **31**) by reaction with 9-fluorenylmethoxycarbonyl chloride in dry pyridine and the 3'-hydroxyl sites reacted with N,N-diisopropylmethoxyphosphonamidic chloride to produce the phosphoramidites (**32**, **33** and **34**) which were characterised by phosphorus NMR against a phosphate standard.

Synthesis of oligonucleotides was then carried out using the PIPME1 synthesis cycle (Append. A., PIPME1), (Table 11) and 5% piperidine in acetonitrile as deblocking reagent . With the final 9-fluorenylmethoxycarbonyl group in place, all amine protection was removed by treatment with a solution of 3% trichloroacetic acid in dichloromethane (10 minutes). The final 5'-protecting group was removed and the oligonucleotide deprotected at the phosphorus and cleaved from the resin using the normal methoxyphosphoramidite ending procedure.(Append. A., depr). All products were analysed by reversed phase and ion exchange HPLC and compared with oligonucleotide standards of the same sequence prepared by routine methods.

In this way $d(A)_8$ synthesis (**m**), $d(GA)_3$ synthesis(**q**), and $d(GT)_3$ synthesis(**o**) were successs-fully prepared in similar yield and purity to the 5'-O-(9-fluorenylmethoxycarbonyl)- N-acyl product obtained previously(Fig. 44 - 52). The synthesis $d(C)_6$ was unsuccessful showing only a tiny amount of product .

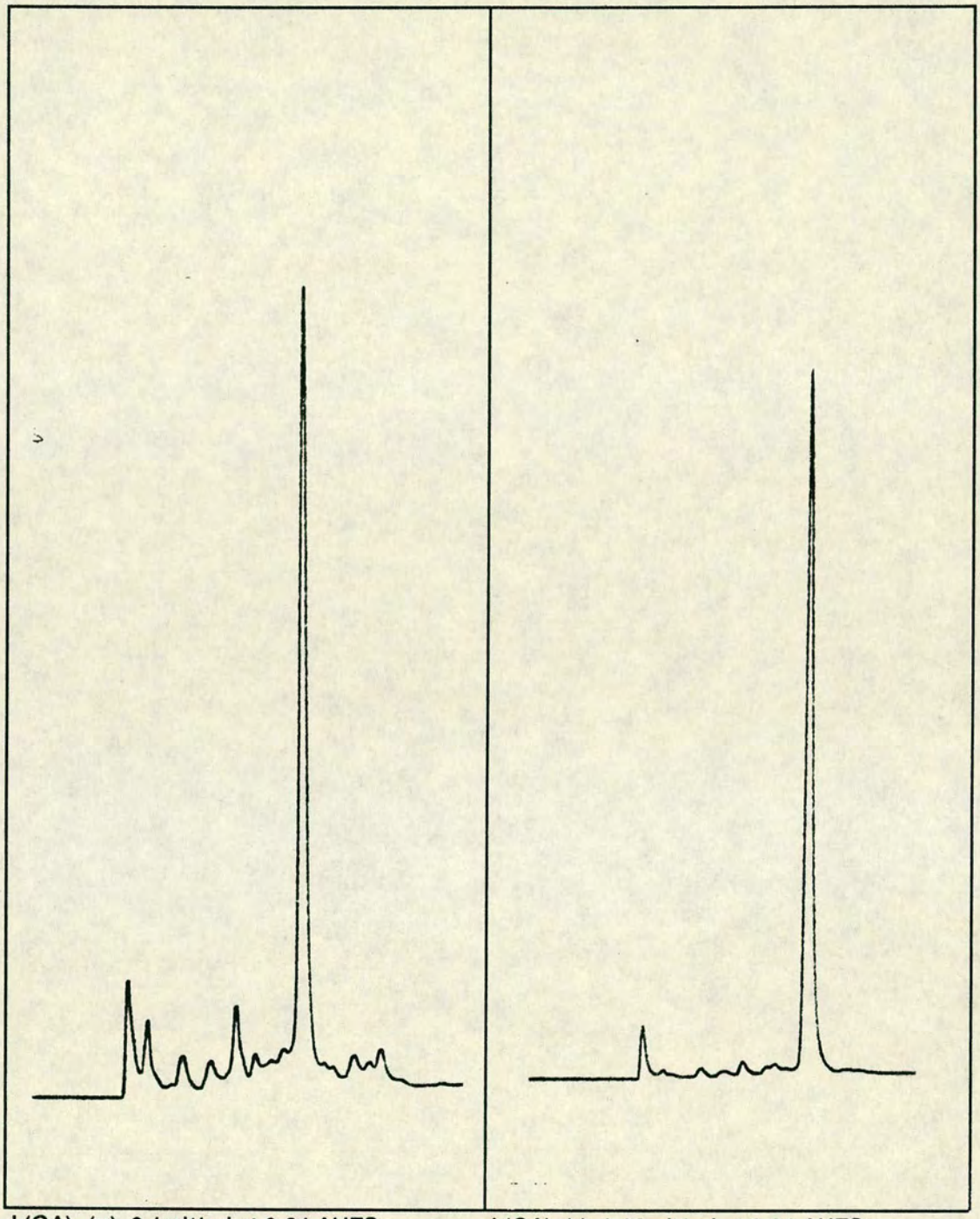
Removal of all protecting groups before cleavage of the oligonucleotide from the support simplifies the post synthetic treatment of the oligonucleotide product, as heating in concentrated



d $(A)_{20}$ (**n**), 0.03ml/1ml at 0.64 AUFS Monitored at 264nm

Ion Exchange HPLC (75% of actual size)

( Figure 43. )



d $(GA)_3$ (**q**), 0.1ml/1ml at 0.64 AUFS

Monitored at 264nm

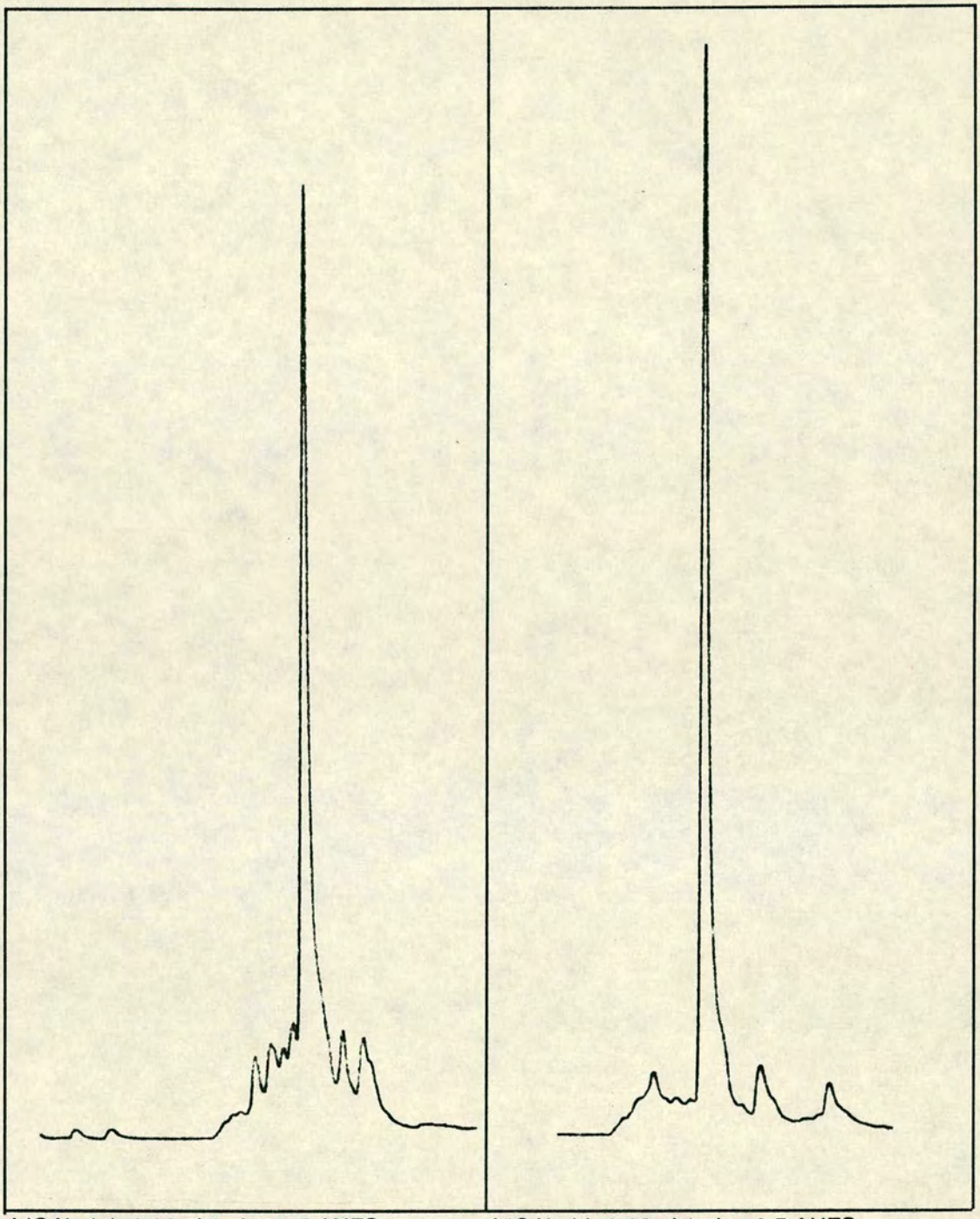
Ion Exchange HPLC

d $(GA)_3$ (**r**), 0.03ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 44. )



d $(GA)_3$ (**q**), 0.08ml/1ml at 0.5 AUFS

Monitored at 264nm

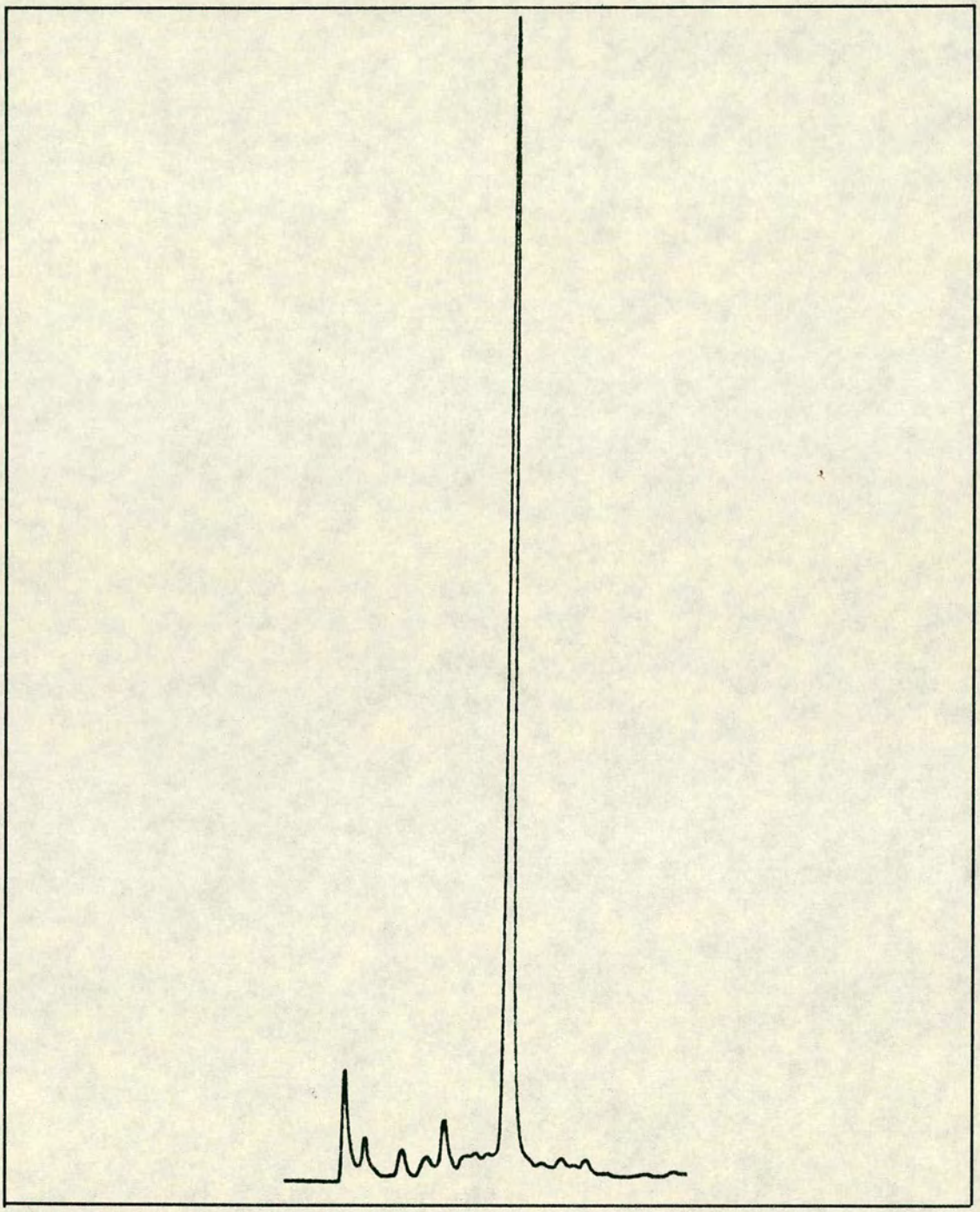
Reversed Phase HPLC

d $(GA)_3$ (**r**), 0.03ml/1ml at 0.5 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 45. )

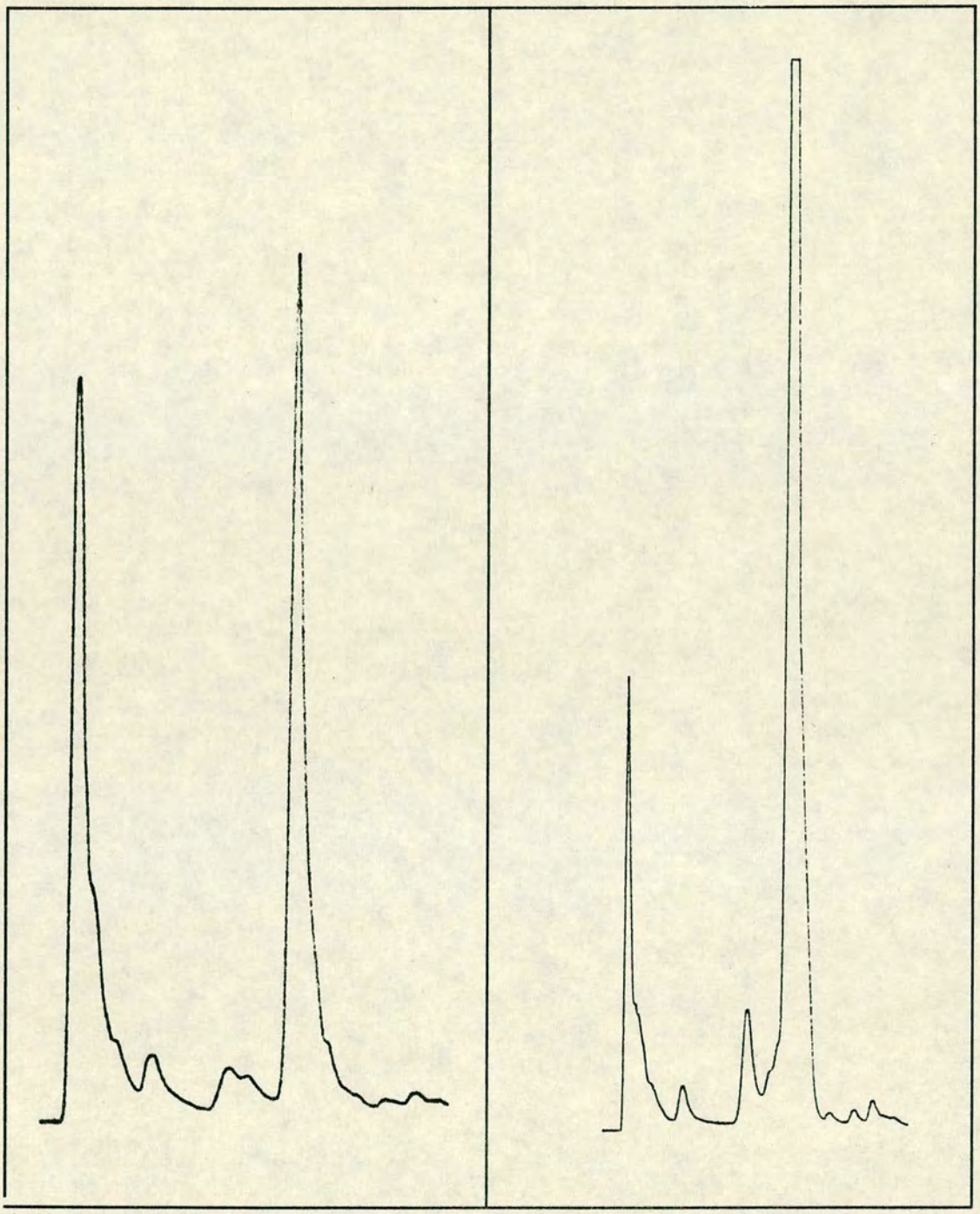


d $(GA)_3$: (**q**) 0.05ml/1ml + (**r**), 0.03ml/1ml mixed sample at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 46. )



d $(A)_8$ (**m**), 0.05ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

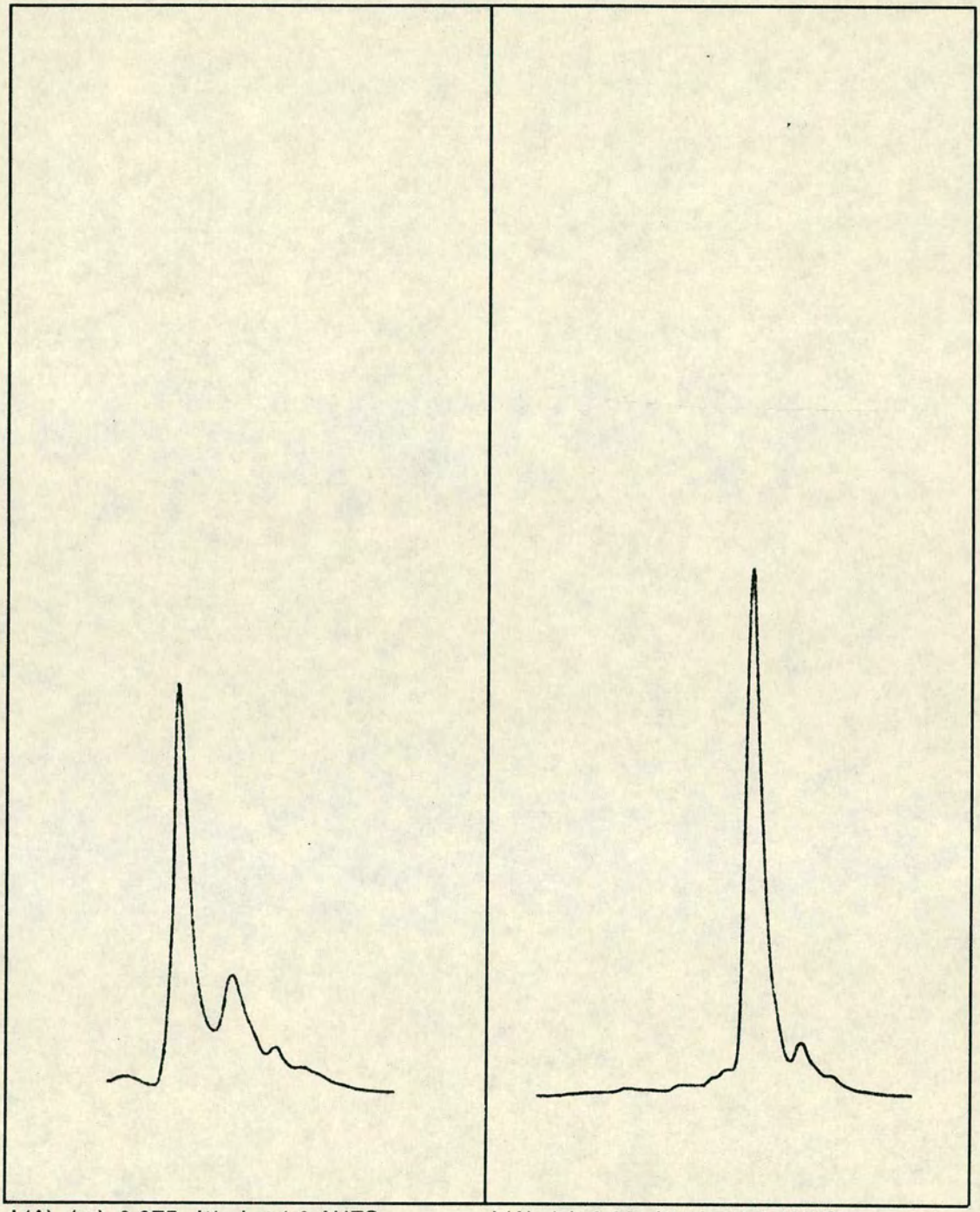
d $(A)_8$ (**e**), 0.05ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

(50% of actual size)

( Figure 47. )



d $(A)_8$ (**m**), 0.075ml/1ml at 1.0 AUFS

Monitored at 264nm

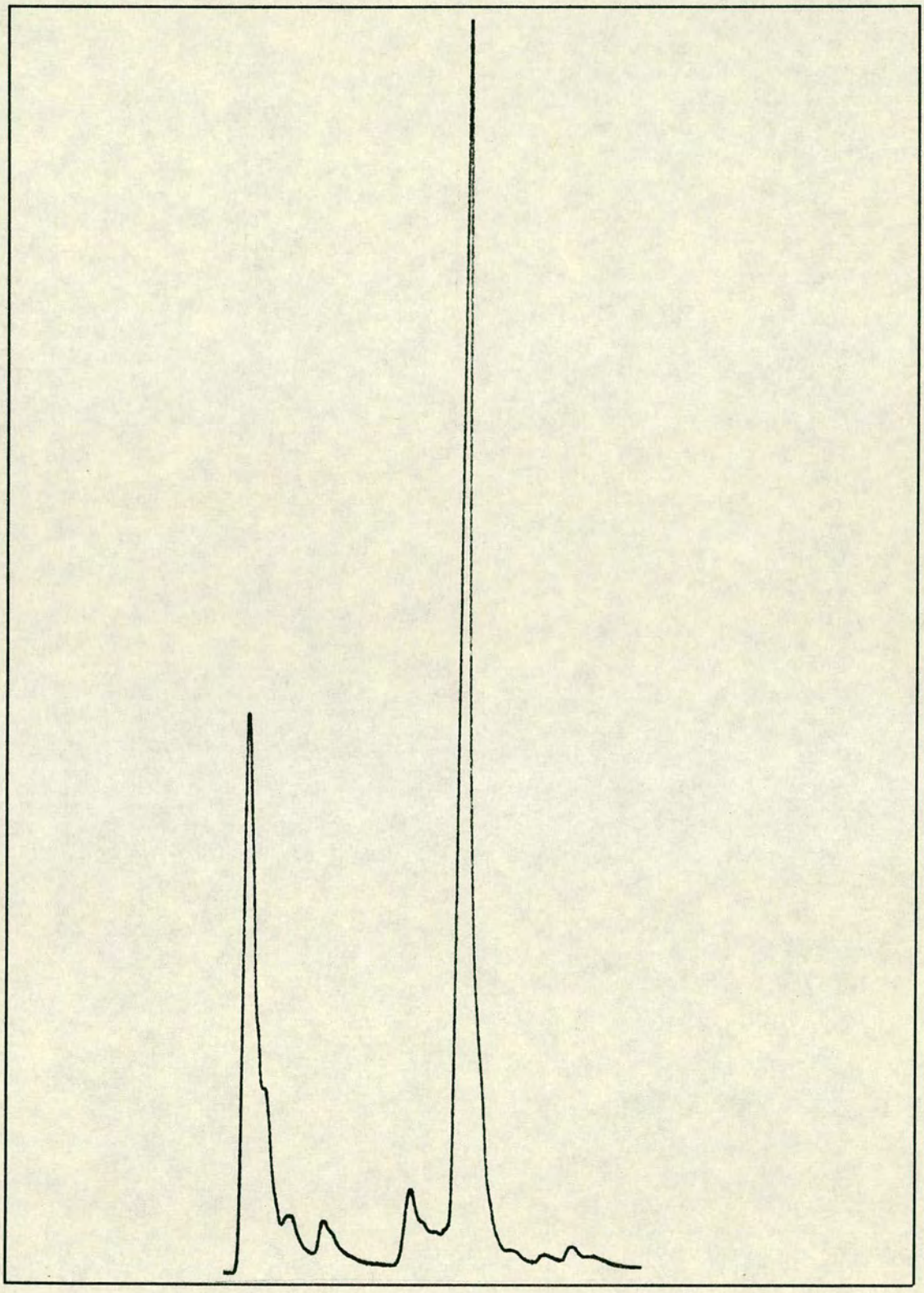
Reversed Phase HPLC

d $(A)_8$ (**e**), 0.02ml/1ml at 1.0 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 48. )

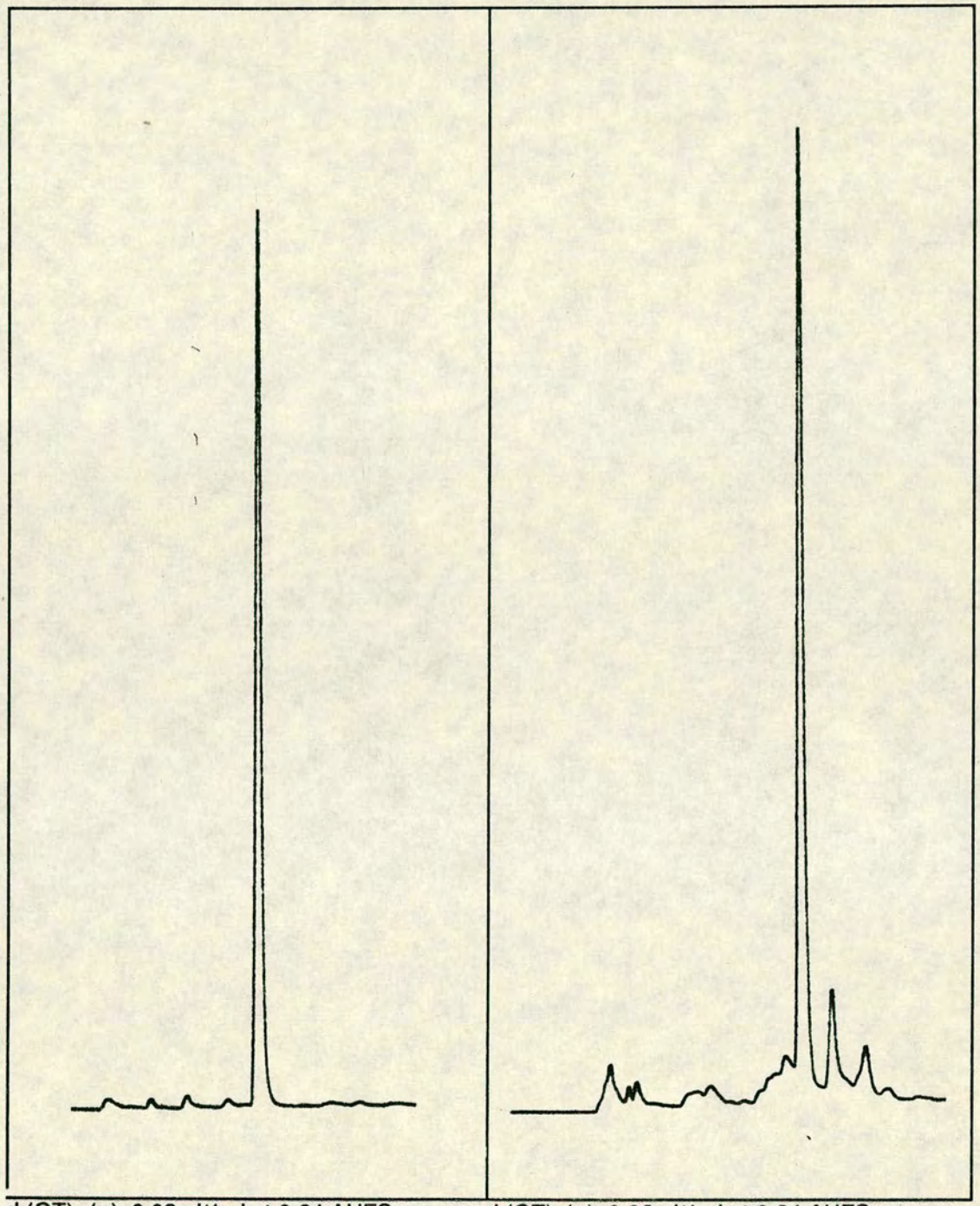


d (A)8 : (**m**), 0.03ml/1ml + (**e**), 0.01ml/1ml mixed sample at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 49. )



d $(GT)_3$ (**p**), 0.03ml/1ml at 0.64 AUFS

Monitored at 264nm

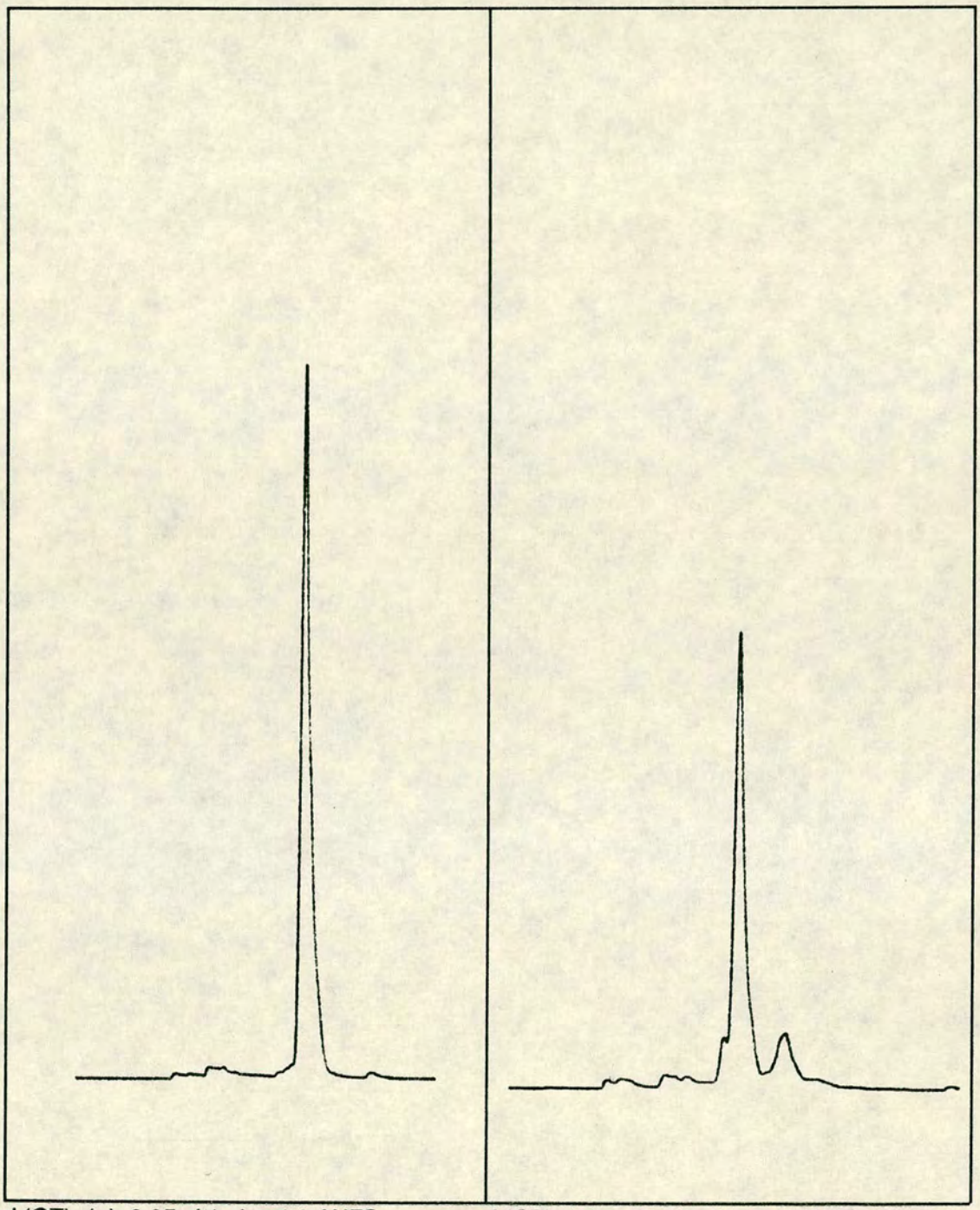
Ion Exchange HPLC

d $(GT)_3$ (**q**), 0.06ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

( Figure 50. )



d $(GT)_3$ (**p**), 0.05ml/1ml at 1.0 AUFS

Monitored at 264nm

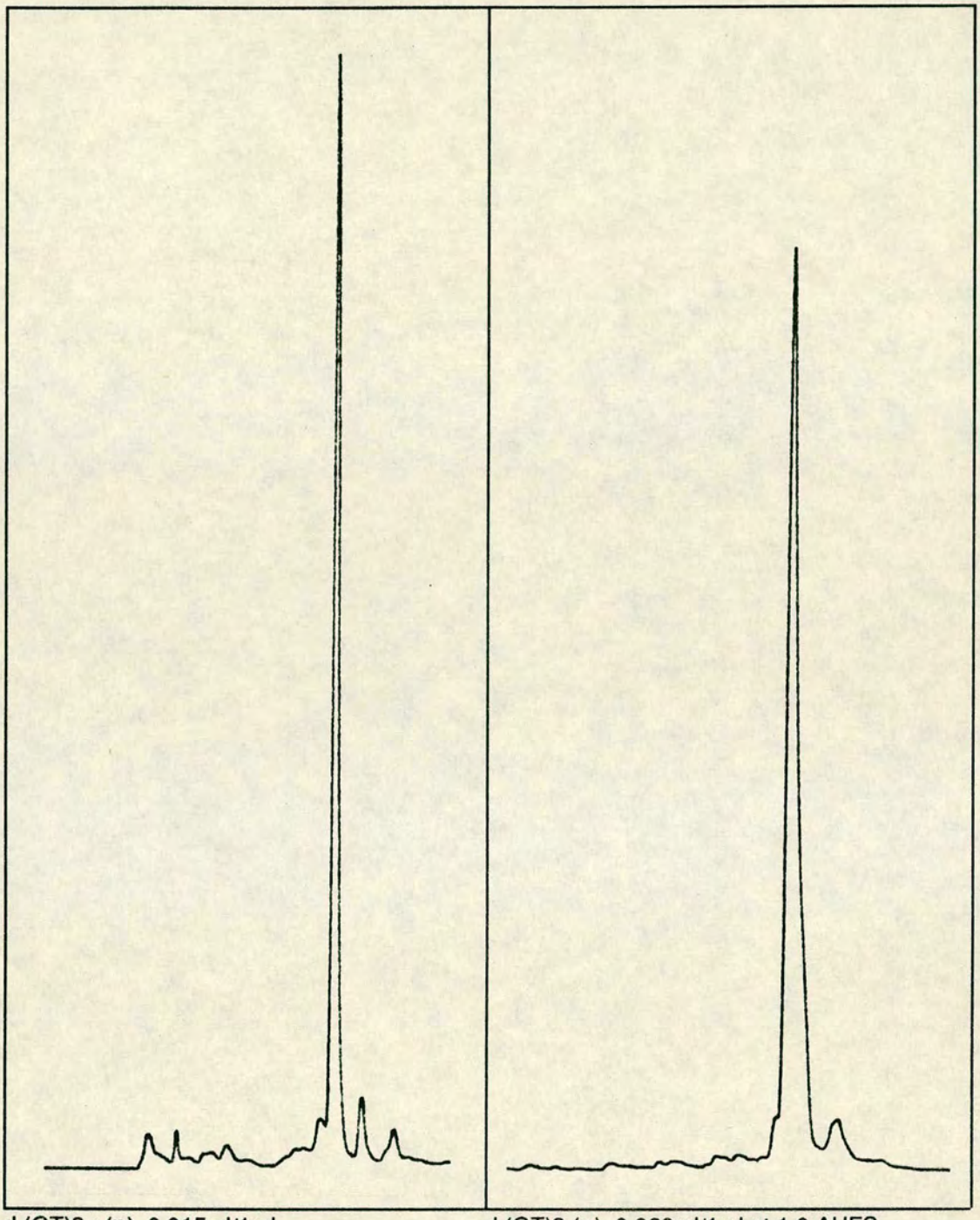
Reversed Phase HPLC

d $(GT)_3$ (**q**), 0.07ml/1ml at 1.0 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 51. )



d (GT)3 : (**p**), 0.015ml/1ml +

(**q**), 0.040ml/1ml at 0.64 AUFS

Monitored at 264nm

Ion Exchange HPLC

d (GT)3 (**q**), 0.060ml/1ml at 1.0 AUFS

(**p**), 0.05ml/1ml at 1.0 AUFS

Monitored at 264nm

Reversed Phase HPLC

( Figure 52. )

ammonia solution is no longer required. Complete deprotection of resin-bound oligonucleotides may also allow the simple preparation of materials for hybridisation experiments .

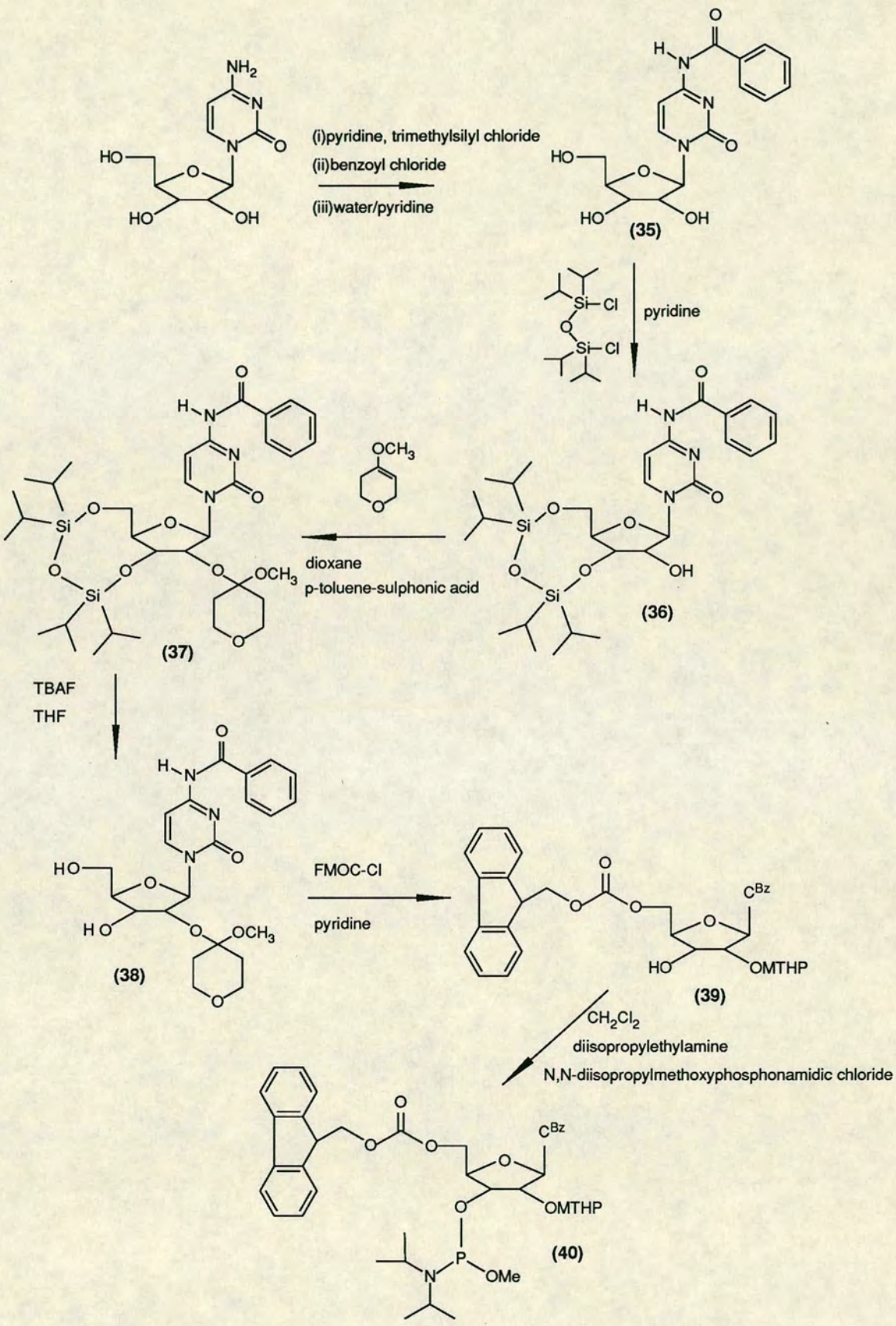
The decamer d( GATGTAGTAT) synthesis(**t**) was synthesised by the above strategy and allowed to remain attached to the controlled pore glass resin. After annealing to d(ACTACATCATACTACATC) synthesis(**u**) at 70°C for 15 minutes, a drop in uv absorbance of the free oligonucleotide of 15% was observed indicating that some hybridisation had taken place. Further samples (in gram quantities) of the T, dG, and dA monomers were prepared for investigation at Pharmacia LKB.

### 2.4 **The 5'-O-(9-fluorenylmethoxycarbonyl) Group in Oligoribonucleotide Synthesis**

A strategy for oligoribonucleotide synthesis was designed, making use of 5'-hydroxyl 9-fluorenylmethoxycarbonyl protection, acyl base protection and 4-methoxytetrahydrpyran-4-yl 2'-protection.The 2'-group is acid labile but stable to bases and therefore should remain intact during both 5'-deblocking steps and acyl group cleavage.

Benzoylcytidine (**35**) was prepared by a transient protection methodology (Scheme 24) and was 3',5'-O- protected with the tetraisopropyldisiloxane group. This silylated derivative (**36**) was then converted to the 2'-acetal (**37**) and, after removal of the tetraisopropyldisiloxyl group with tetrabutylammoniumfluoride in THF, the 5'-hydroxyl position was protected with a 9-fluorenylmethoxycarbonyl group (Scheme 24) (**39**). This nucleoside was then converted into a 3'-O-N,N-diisopropylmethoxyphosphoramidite (**40**) by reaction with N,N-diisopropylmethoxyphosphonamidic chloride in the presence of diisopropylethylamine (Scheme 24).

A short oligonucleotide $r(C)_6$ (**v**) was synthesised using synthesis cycle (RNA2P)(Table 13). Deblocking of the 5'-hydroxyl was exactly as with other 9-fluorenylmethoxycarbonyl syntheses but a longer coupling time is required in oligoribonucleotide synthesis.Phosphate methyl groups were removed by treatment with thiophenol and triethylamine in dioxane and the oligonucleotide cleaved from the resin using standard methodology (end procedure "depr " Appendix A.). The benzoyl protecting groups were cleaved by treatment with concentrated aqueous ammonia solution at 55°C for 5.5 hours and the methoxytetrahydropyranyl groups were cleaved with 0.01M hydrochloric acid at pH 2 for 1 hour.


(i)pyridine, trimethylsilyl chloride
(ii)benzoyl chloride
(iii)water/pyridine
(35)
pyridine
dioxane
p-toluene-sulphonic acid
(36)
(37)
TBAF
THF
(38)
FMOC-Cl
pyridine
(39)
$CH_2Cl_2$
diisopropylethylamine
N,N-diisopropylmethoxyphosphonamidic chloride
(40)

( Scheme 24. )

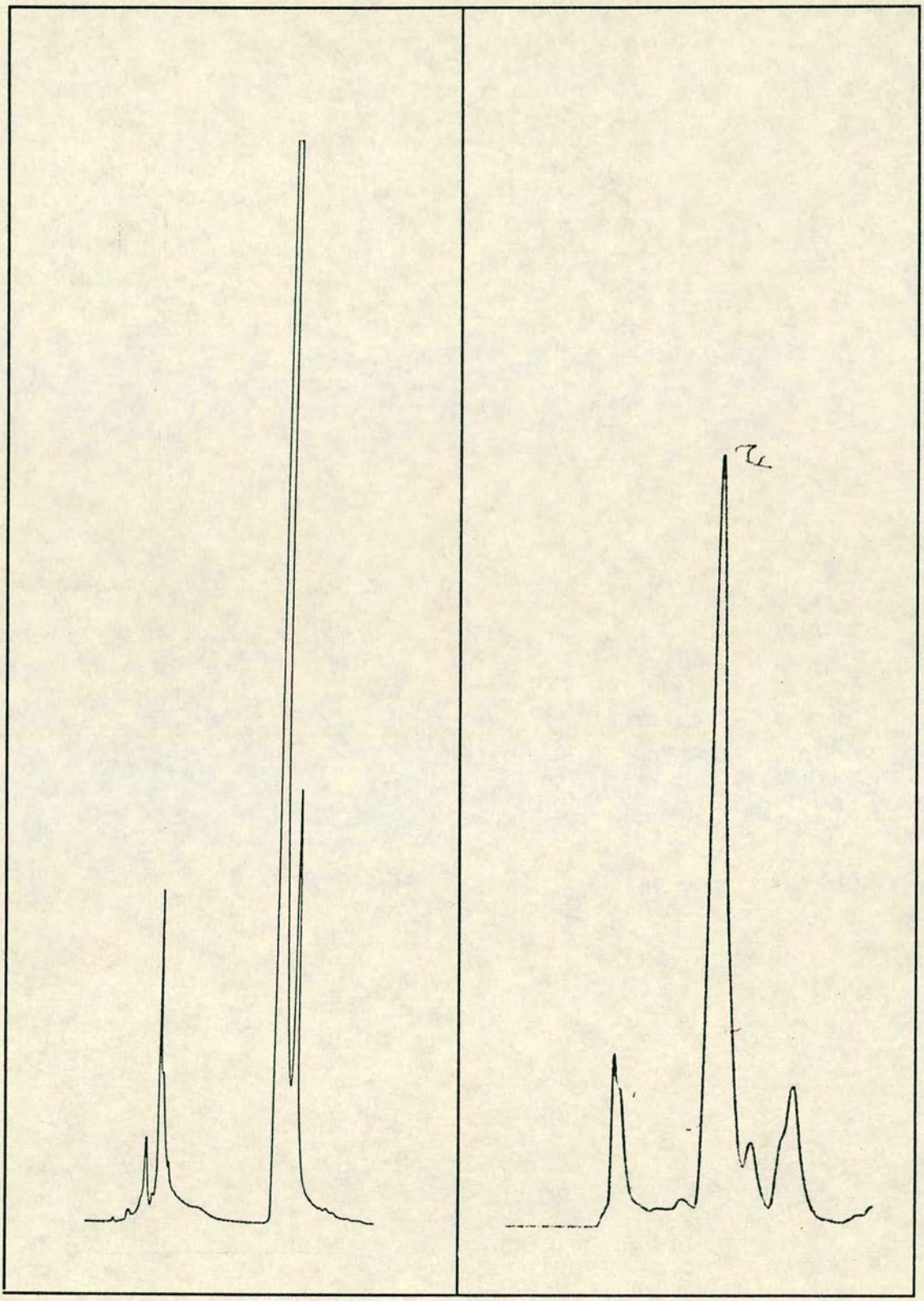
| Operation | Reagent/solvent | Time/s |
|---|---|---|
| wash | acetonitrile | 30 |
| deblock 5' | 5% piperidine in acetonitrle | 180 |
| wash | acetonitrile | 30 |
| wash | anhydrous acetonitrile | 60 |
| coupling step | 0.1M phosphoramidite in acetonitrile<br>0.5M tetrazole in acetonitrile | 300 |
| capping step | acetic anhydride/lutidine/THF 1/1/8<br>17.6% N-methylimidazole in THF | 40 |
| oxidation | 0.1M $I_2$/water/pyridine/THF<br>2/20/80 | 50 |

( Table 13. )

The product was analysed by ion exchange and reversed phase HPLC both before and after removal of the methoxytetrahydropyranyl group (Fig. 53, 54).

The HPLC chromatograms indicated that the product was of similar purity to the deoxyoligonucleotides synthesised using 9-fluorenylmethoxycarbonyl protection. This would therefore seem to be a viable method of oligoribonucleotide synthesis (as later shown by Gait and coworkers[68]). However our product still showed a significant peak after the product peak in the ion exchange HPLC trace and it was feared that in longer syntheses this might be difficult to separate from the product.

In addition to this the 5,6-dihydro-4-methoxy-2H-pyran starting material for 2'-O-protection must be used in large excess for a successful reaction to take place.It can be synthesised in a 3 step process from 1,5-dichloropentan-3-one[110,111] (Scheme 25) but the yields are relatively low and synthesis is time consuming. The compound is available commercially but the price reflects



r $(C)_6$ (v), before removal of MTHP

Monitores at 280nm

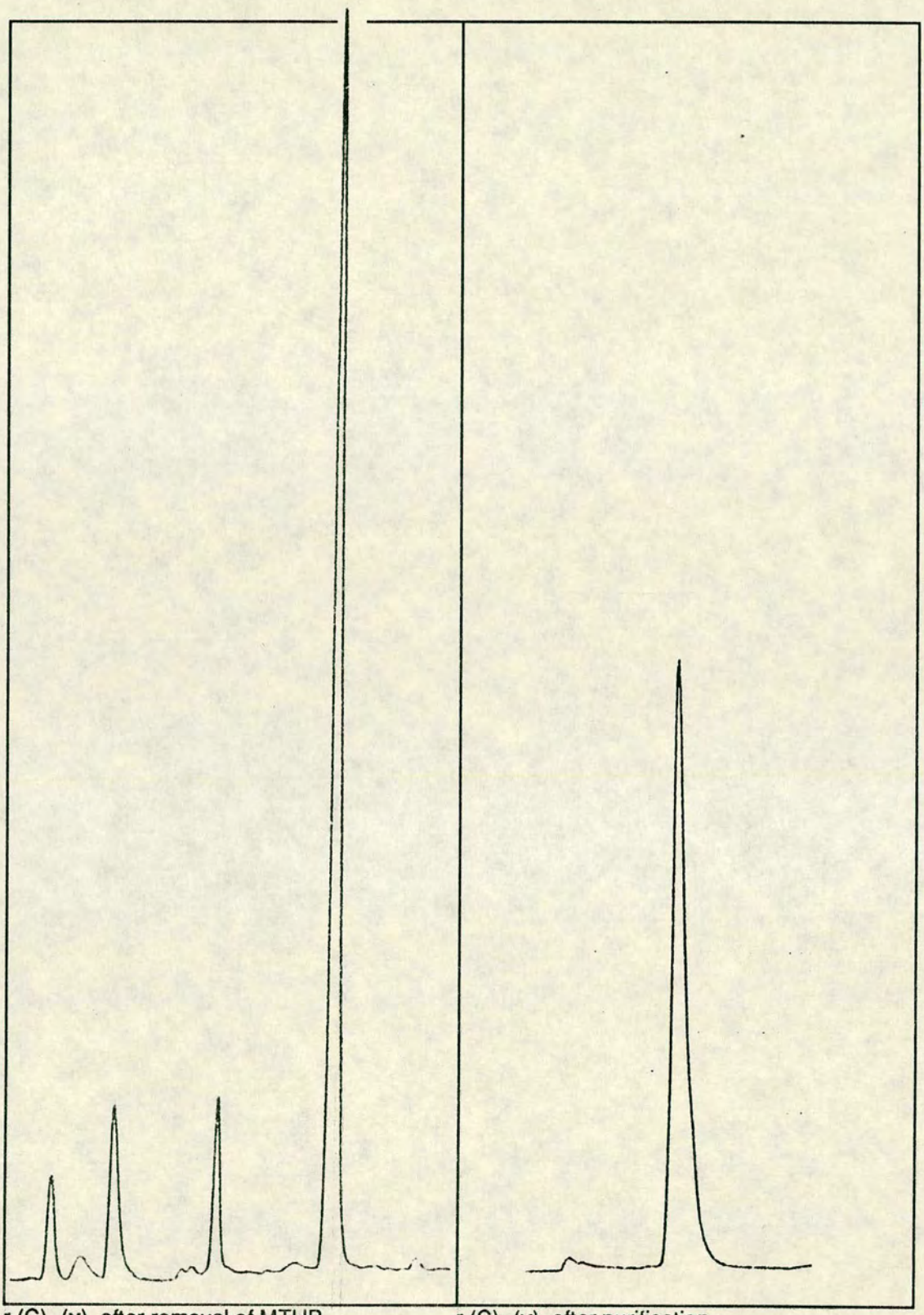
Reversed Phase HPLC

r $(C)_6$ (v), after removal of MTHP

Monitored at 280 nm

Reversed Phase HPLC

( Figure 53. )



r $(C)_6$ (v), after removal of MTHP

Monitored at 280nm

Ion Exchange HPLC

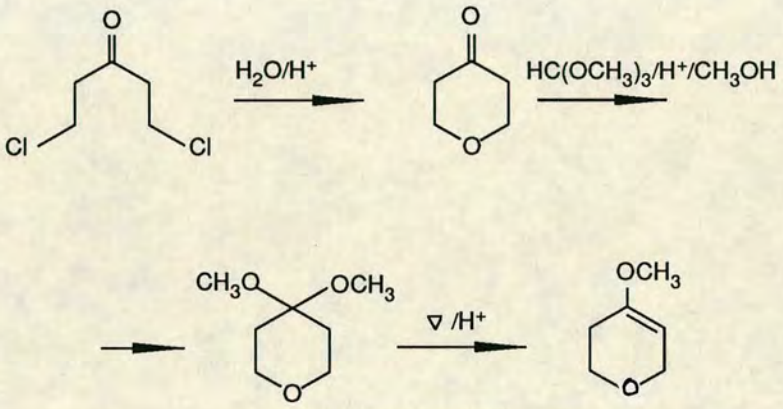
r $(C)_6$ (v), after purification

Monitored at 280nm

Reversed Phase HPLC

( Figure 54. )

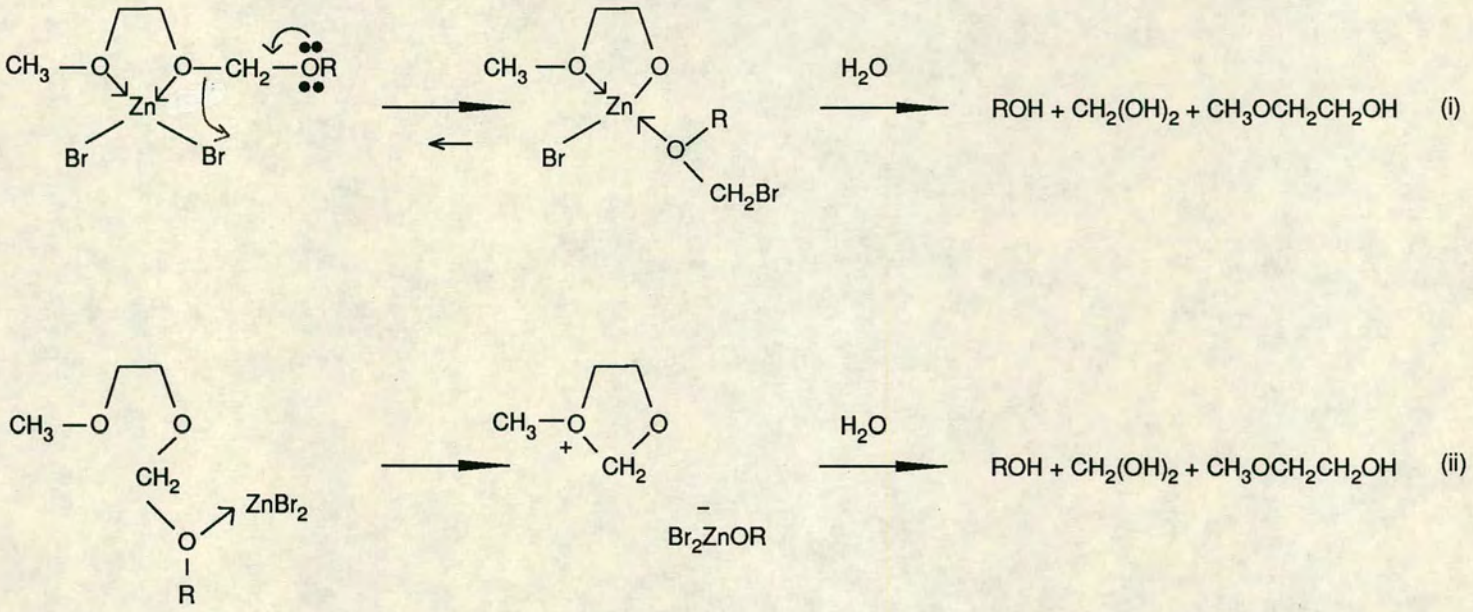
the difficult synthetic route. In a single protection reaction this cost/time factor would not be crucial, but in nucleotide preparation a further 3 reactions follow in the general synthetic scheme( scheme 24) and the yield of the second of these, the 9-fluorenylmethoxycarbonyl addition is only of the order of 50%. Hence this route leads to 50% destruction of expensive protecting groups and is therefore unlikely to be viable for commercial or large scale oligoribonucleotide synthesis. As this strategy was then published by Gait and coworkers[68] it was decided to investigate other 2'-protecting groups.



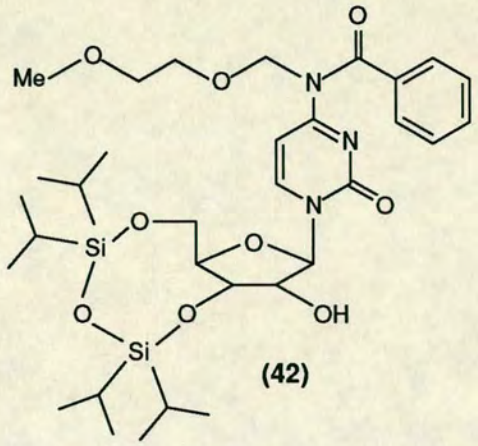

( Scheme 25. )

Reaction of methoxyethoxymethyl triethylammonium chloride(MEM $NEt_3Cl$) with uridine had previously been reported.[84] The MEM group had previously been used to protect both 2'-ribose hydroxyl and N(3) of the base in the preparation of polyU but had not been applied to other nucleosides. The methoxyethoxymethyl ether originally designed by Corey and coworkers[112] is stable to basic hydrolysis and is cleaved by the action of Lewis acid catalysts like $ZnBr_2$ . Two mechanisms of cleavage were proposed[112] (Fig. 55). The stability of this protecting group may also allow the use of a wide range of acid or base labile 5'-hydroxyl protecting groups.

Reaction of 4-N-benzoyl-3',5'-O-(tetraisopropyldisiloxanedi-3-yl)cytidine with $MEMNEt_3Cl$ (**41**) in refluxing ether gave a crystalline product (**42**) with a single MEM group attached at the N(4) amide position (Fig. 56). This was confirmed by a combination of proton NMR and mass spectrum data. Upon treatment with ammonia solution at 60°C overnight the uv activity of this nucleoside was lost indicating degradation of the heterocyclic base had occurred.



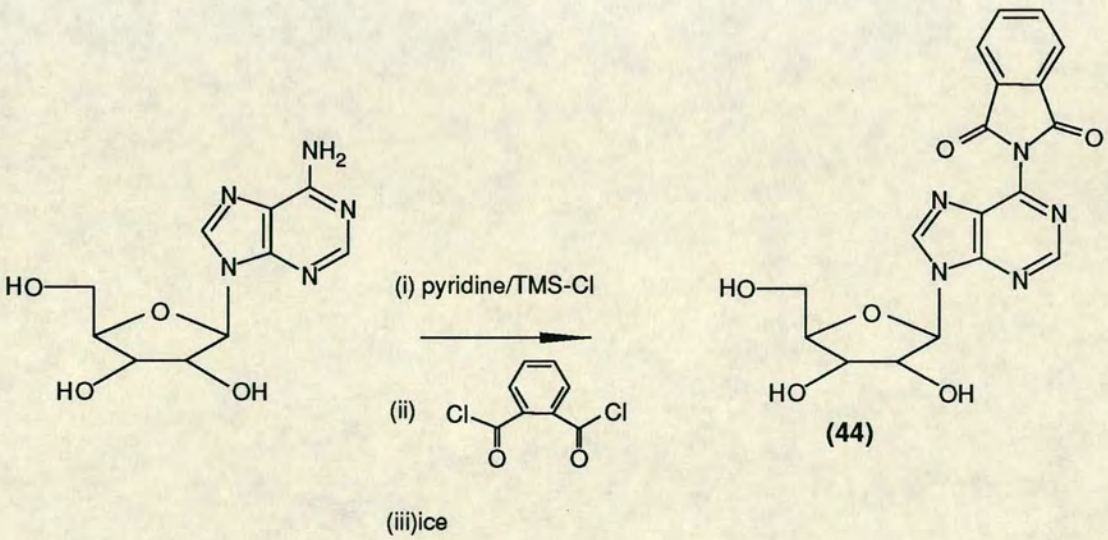

( Figure 55. Proposed mechanism of MEM cleavage )




**(42)**

( Figure 56. )

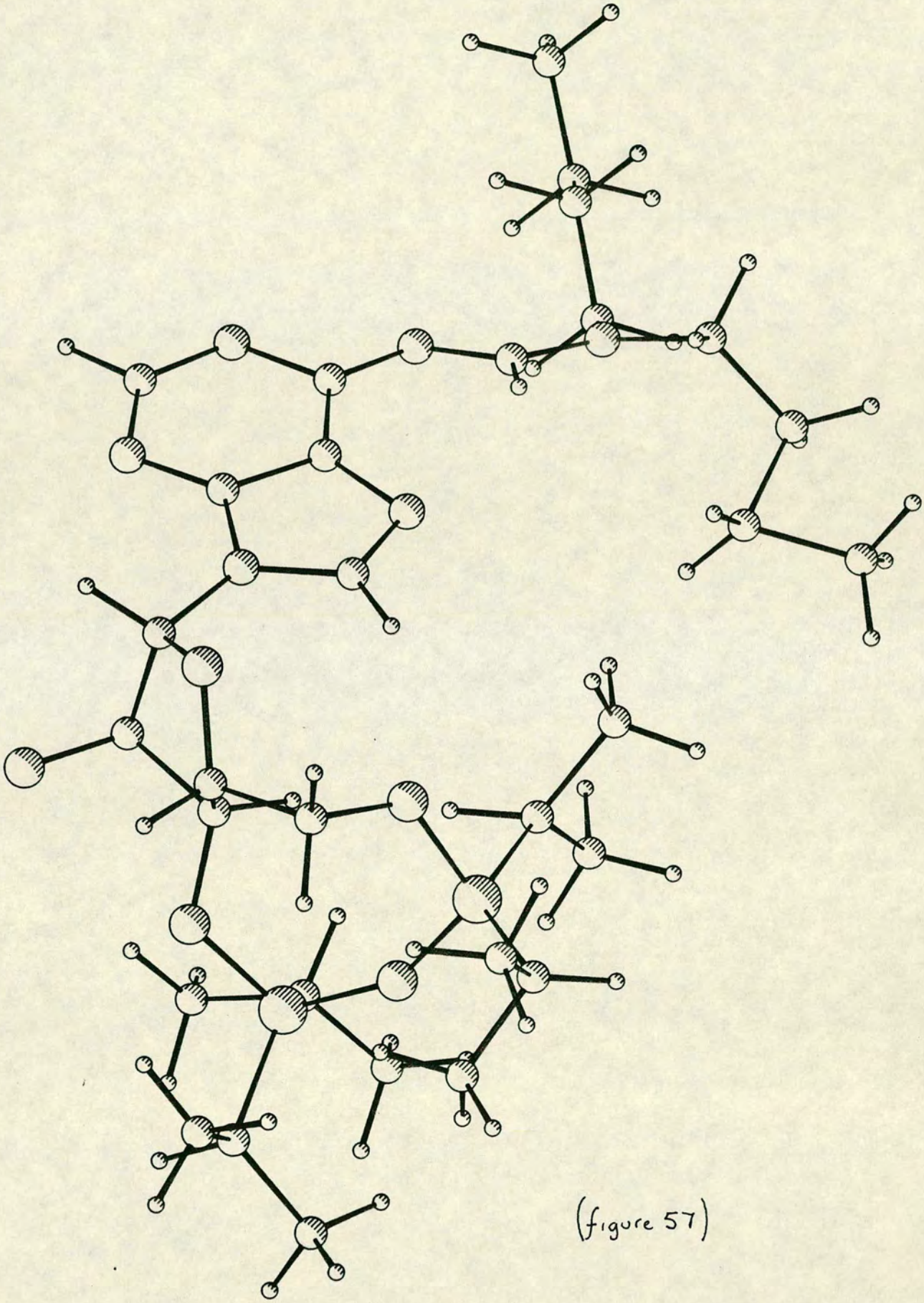
A method of completely preventing the addition of a MEM group to the base was needed.The possibility of using cyclic diacyl groups to protect both reactive sites on the N(6) amino group of adenine was investigated; 6-N-phthaloyladenosine (**44**) (Scheme 26) and 6-N-phthaloyl-3',5'-O-tetraisopropyldisiloxanedi-3yl-adenosine (**45**) were synthesised. Synthesis of 4-N-phthaloylcytidine (**43**) was also attempted but led to only a 10% yield. The diacyl groups were found to be unstable to fluoride ions and thus incompatible with the tetraisopropyl protection.




**(44)**

( Scheme 26. )

Alternative "double protection" at the amino-group of adenosine can be achieved with the use of dialkylforamidine groups[51]. 6-N-di-n-butylformamidine-3',5'-O-tetraisopropyldisiloxanedi-3-yladenosine (**49**) was easily prepared by reaction of 3',5'-tetraisopropyldisiloxanedi-3-yl-adeno-



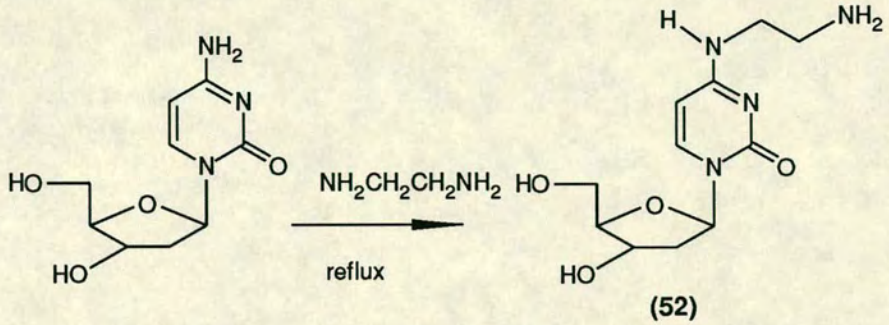
(figure 57)

sine (**48**) with di-n-butyldimethylacetal (**47**) in dichloromethane at room temperature and this compound was found to be stable to fluoride ions.The protected product was a mixture of 2 geometric isomers at the formamidine N-C double bond. It was possible to crystallise the major isomer of the product of this reaction from hexane, the X-ray structure of which was determined by Dr G.A.Leonard. This clearly showed the trans geometric isomer of the formamidine group as well as the position of the 2'-hydroxyl function which appears to be unhindered by the tetraisopropyldisiloxyl group (Fig. 57).No successful addition of MEM to this compound was achieved, under a variety of conditions and it may be concluded that activated MEM derivatives are a little too unreactive to react with the 2'-secondary hydroxyl group in silylated nucleosides.

### 2.5 **Fluorescent Labelling of Oligonucleotides with the 2,4-Dinitrophenyl Group**

Antibodies specific for the dinitrophenyl moiety are well known and have been used to study proteins and peptides. Sanger's reagent, 2,4-dinitrofluorobenzene reacts with primary amines of side chains and alpha-amino groups within the peptide. Dinitrophenyl labelling of oligonucleotides has also been reported but only in solution phase reactions. No direct solid phase labelling strategy has yet been reported. Solution phase labelling involves the reaction of 2,4-dinitrofluorobenzene with an oligonucleotide carrying a primary aliphatic amine group. The amine group may be introduced by modification of a nucleoside base. A modified nucleotide monomer for oligonucleotide synthesis has the advantage of allowing multiple label addition within the oligonucleotide structure but may involve a lengthy synthetic preparation making it unsuitable for routine use. In a second approach to amino group introduction, a simple aliphatic amino alcohol is functionalised to produce a monomer suitable for oligonucleotide synthesis, carrying a protected primary amine site. These nucleotide analogue monomers have the advantage of being simple to prepare from inexpensive starting materials.

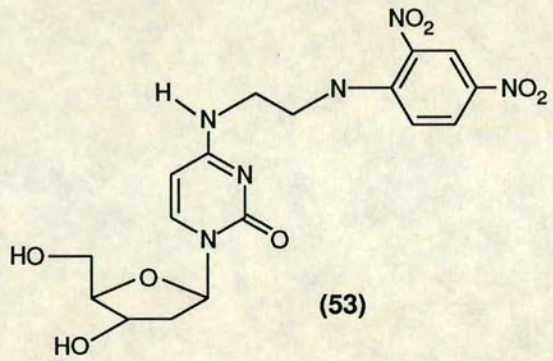
2,4-Dintrofluorobenzene does not react with the N(4) amino group of 5'-O-(4,4''-dimethoxytrityl)-2'-deoxycytidine (**50**) and so the initial strategy investigated was to modify this N(4)-site to carry a short alkyl linker ending with a primary amino-group. 2'-Deoxycytidine was refluxed with ethylenediamine and the result was an insoluble oil (**52**) (Scheme 27) of high boiling point which




**(52)**

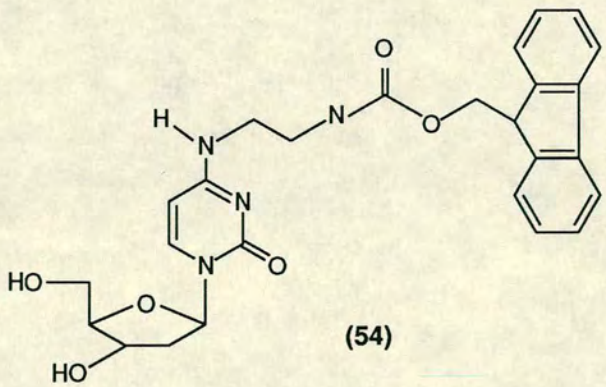
( Scheme 27. )

was difficult to purify and was therefore only partially characterised. The product (**54**) of the reaction of this N(4) modified nucleoside with 9-fluorenylmethoxycarbonyl succinimide (Fig. 59) had the desired peak in the FAB mass spectrum. The oil (**52**) also reacted vigorously with 2,4-dinitrofluorobenzene(**53**) (Fig. 58). Both the FMOC and the 2.4-dinitrophenyl derivatives were equally insoluble.




**(53)**

( Figure 58. )

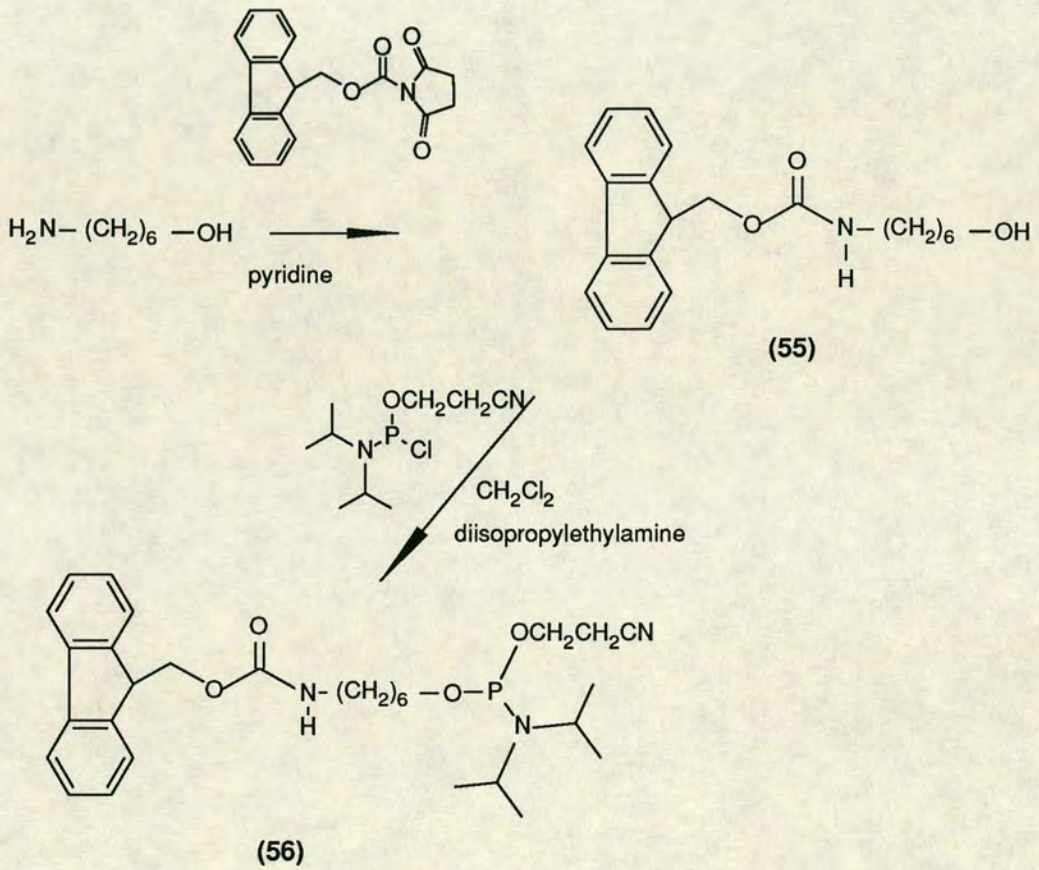



**(54)**

( Figure 59. )

In an attempt to produce a labelled amine, for use in base modification reactions, ethylenediamine was treated with 1 equivalent of dinitrofluorobenzene in ether and an insoluble yellow solid (**51**) was produced, thought to be a hydrofluoride salt. Attempts to solublise this salt in organic solvents by washing with 2M sodium hydroxide solution failed. The mass spectrum of this compound showed a signal at m/z = 227 corresponding to the expected molecular ion.

A reagent with only one primary amine group was therefore selected, 6-aminohexan-1-ol, and

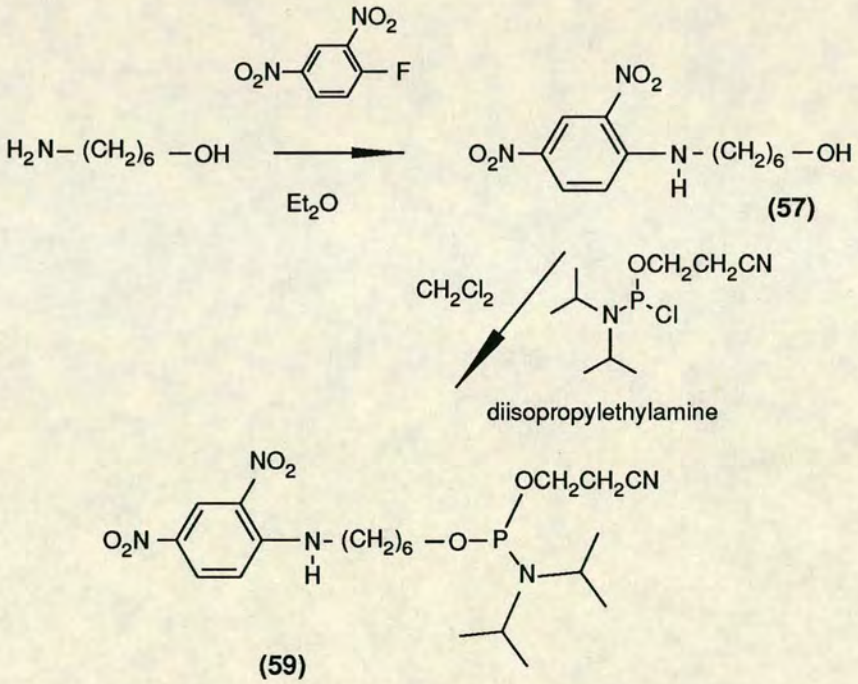



( Scheme 28. )

a nucleotide analogue approach was now attempted. This methodology has the advantage of simplicity over the base modification approach. Readily available amino-alcohols can be N-protected easily and converted by conventional chemistry to monomers suitable for oligonucleotide synthesis. A monomer (**56**) was prepared with the amine protected with the 9-fluorenylmethoxycarbonyl group (Scheme 28). This cyanoethylphosphoramidite was coupled to free hydroxyl of thymidine long chain alkyl-amine controlled pore glass resin and after oxidation of the

phosphoramidite the FMOC group was removed with piperidine solution to give a resin with a primary amine group attached .This was shown by a positive ninhydrin test[113,2] for amines and by the fact that the resin turned bright yellow on treatment with dinitrofluorobenzene.

The FMOC-amino-hexyl monomer was therefore used in oligonucleotide synthesis, coupled at the 5'-end in the sequence d(FMOC-aminohexanol-CGCGAATTCGCG) synthesis(**w**) .The FMOC group was removed with 5% piperidine in acetonitrile (5 minutes) and the resin soaked in 10% dinitrofluorobenzene solution (v/v) in dichloromethane for 1 hour. The oligonucleotide was then cleaved from the resin, deprotected as normal and the product analysed by HPLC. No oligonucleotide was visible by HPLC at all, even when reversed phase analysis was carried out with 40% acetonitrile solution. The reason for this failure was not clear and so it was considered to be simpler and easier to change to a different approach.




( Scheme 29. )

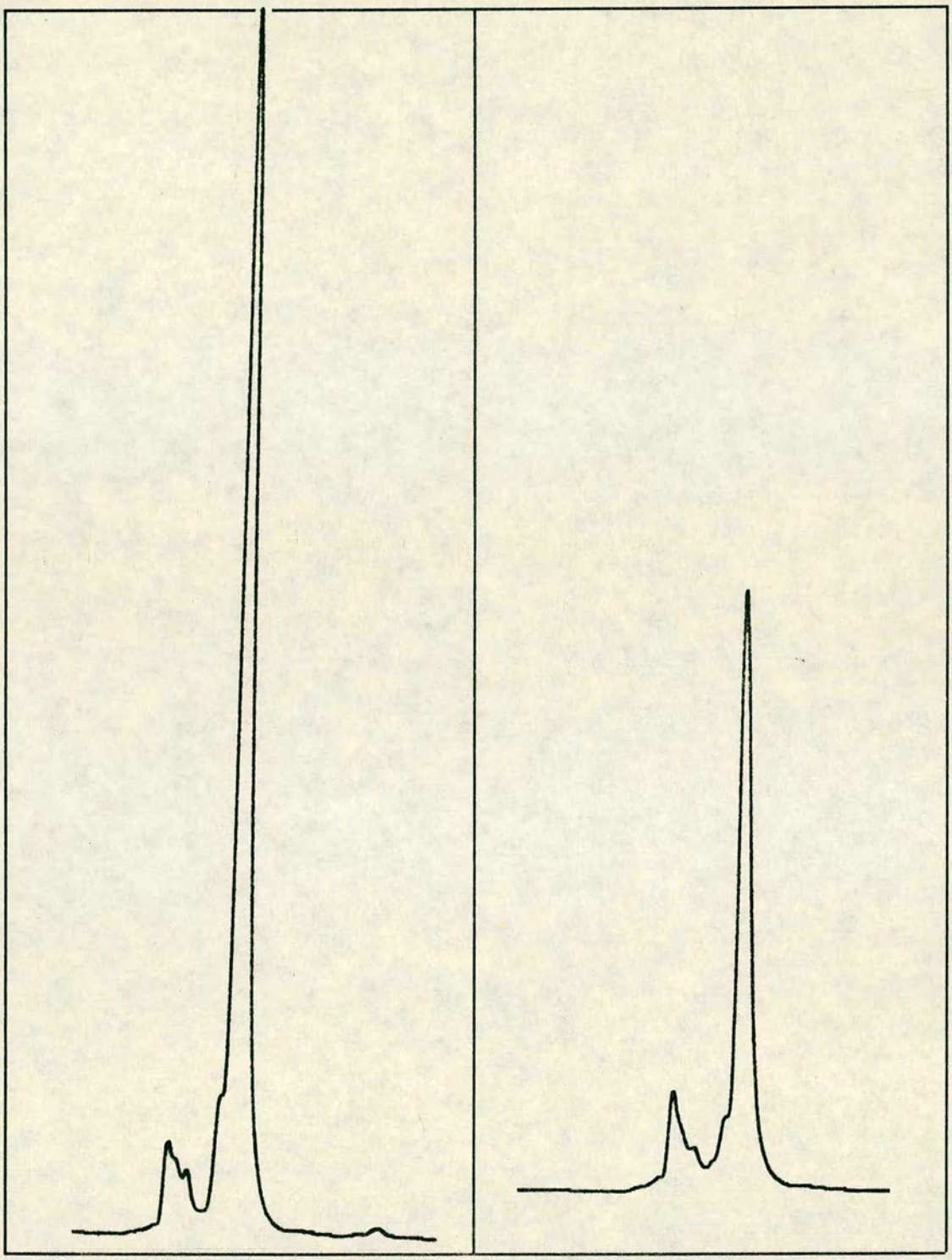
Amino-hexanol was reacted directly with dinitrofluorobenzene to give compound (**57**) (Scheme 29 ). It was found that the secondary amine produced in this reaction could not be further protected by benzoylation with benzoyl chloride in pyridine and it was therefore concluded that the secondary amine would probably be inert during oligonucleotide synthesis. The alcoholic function

was converted to the N,N-diisopropylcyanoethoxyphophoramidite (**59**) with cyanoethoxy-N,N-diisopropylchlorophosphine in the presence of diisopropylethylamine (Scheme 29) and the phosphorus purity of this monomer was confirmed by $^{31}P$ NMR. After flash silica gel chromatography the product was isolated as an oil, soluble in acetonitrile and stable at -20°C for at least 1 month. This monomer (**59**) was then used directly in oligonucleotide synthesis (cycle ssce103a, Table 8 ). The 13mer d(DNP-CGC GAA TTC GCG) synthesis(**x**) was synthesised and deprotected using standard reagents and cycles to give a pure labelled oligonucleotide in good yield. The label was found to be stable to treatment with concentrated ammonia solution at 60°C for at least 48 hours when monitored by HPLC (Fig. 60, 61). Digestion of the oligonucleotide with snake-venom phosphodiesterase and alkaline phosphatase, at 37°C at pH 8,8 in tris buffer, revealed the expected four nucleosides and 6-N-(2,4-dinitrophenyl)-aminohexan-1-ol when analysed by reversed phase HPLC . The effect of the label on hybridisation was investigated by means of DNA melting study by Dr. T.Brown. The labelled oligonucleotide was found to have a melting temperature (70°C) identical to the native 12mer(70°C) indicating that the label neither prevents hybridisation (melting temperature would drop) or intercalates (which might be expected to raise the melting temperature).

A number of labelled oligonucleotides were prepared by the same methodology d(DNP-CCT TTA TAG AAG CGT TCT CGC CCA GCC GCT GCC) synthesis(**y**) was used in a radioimmune competitive binding assay and in a hybridisation test, again showing that the label did not prevent hybridisation.

The sequences d(DNP-CCT CAC AGA GCA AGA TAA GGA), d(DNP-AAG CTT GCA CAA TGC CAA AAA ACA G), and d(DNP-CTC GAG TAT GCC GAG ACC CCT AAT C) syntheses(**z**) were successfully used as primers for polymerase chain reaction by R.W.Honess at the National Institute for Medical Research, Mill Hill, London, and the PCR products were identified by antibody binding which was extremely efficient (preliminary results).

This synthetic methodology provides a simple, inexpensive, reliable route to chemically labelled oligonucleotides which are especially easy to purify by HPLC. Reversed phase HPLC gives excellent separation between non-labelled failure squences and the more lipophilic labelled



Oligonucleotide (**y**), at 0.125 AUFS

0.1ml (after 22 hours in ammonia at 60°C)

Monitored at 290nm

Reversed Phase HPLC

Oligonucleotide (**y**) at 0.125 AUFS

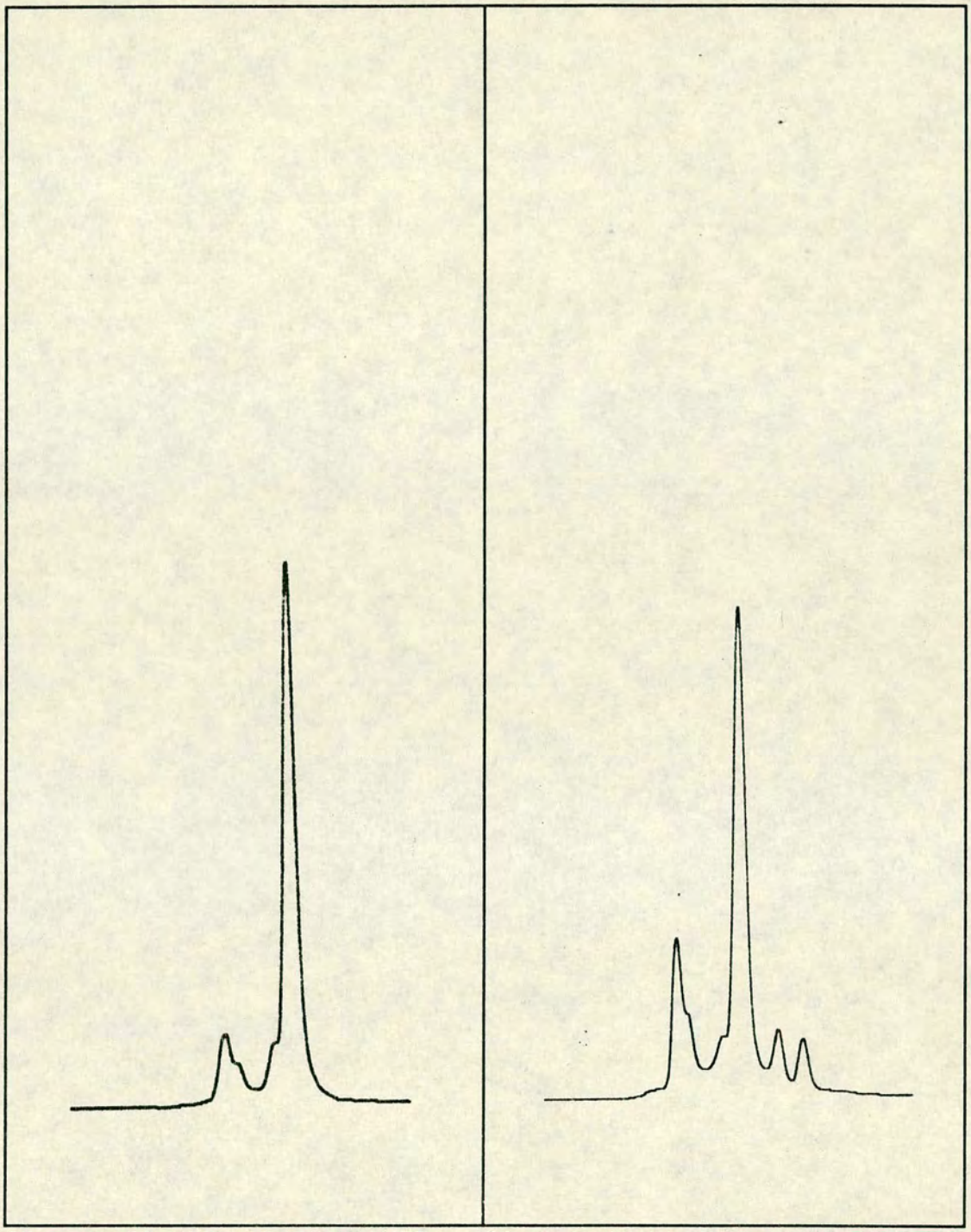
0.1ml (after 22 hours in ammonia at 60°C) and

0.1ml untreated

Monitored at 290nm

Reversed Phase HPLC

( Figure 60. )



Oligonucleotide (**y**), 0.1ml at 0.125 AUFS

( After 40 hours in ammonia at 60°C )

Monitored at 290nm

Reversed Phase HPLC

Oligonucleotide (**y**), 0.1ml at 0.125 AUFS

( After 1 week in ammonia at 60°C )

Monitored at 290nm

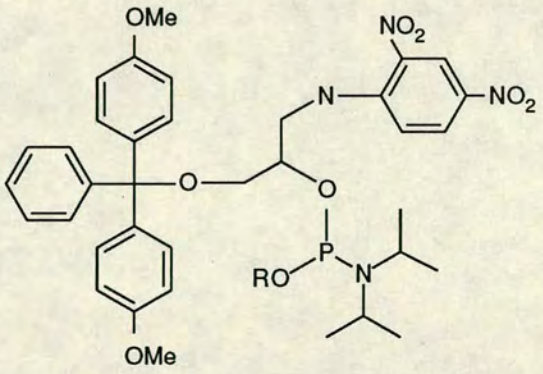
Reversed Phase HPLC

( Figure 61. )

product which is eluted at higher acetonitrile concentration. The DNP-labelled oligonucleotide requires no special handling relative to a non-labelled oligonucleotide and the labelled phosphoramidite monomer can be treated exactly the same as a nucleoside phosphoramidite. In addition no post solid-phase synthetic procedures are required.

The above method is limited to the addition of a single label which effectively blocks the 5'-end of the oligonucleotide chain. An amino-diol, however, could be used to produce an analogue capable of multiple additions at any point in oligonucleotide (Fig. 62). Aminopropan-2,3-diol was selected as a readily available starting material and reacted with dinitrofluorobenzene in methanol. The yellow crystalline product was then protected at the primary hydroxyl site by reaction with 1 equivalent of 4,4'-dimethoxytrityl chloride in anhydrous pyridine.

In the future this compound will be converted to the phosphoramidite at the secondary hydroxyl site and then used in oligonucleotide synthesis to produce multiply labelled products. Although the spacer between the 2,4-dinitrophenyl group and the oligonucleotide is short, if required other compounds could be produced with longer chains to facilitate antibody binding. However there is at present no evidence that a long spacer arm between the DNP-group and the oligonucleotide is necessary.




( Figure 62. )

# Experimental

## Experimental Contents

# Materials and Methods

### 3.1 **Purification of Solvents**

Commercially available solvents were used without further purification, unless otherwise stated. Anhydrous pyridine, diisopropylethylamine and triethylamine were obtained by heating under reflux with potassium hydroxide pellets, followed by distillation. Pyridine was stored under nitrogen in sealed bottles. Dichloromethane was dried by refluxing with calcium hydride followed by distillation and was stored over pre-dried molecular sieves. Tetrahydrofuran (THF) was purified by heating at reflux with sodium and benzophenone, under nitrogen, followed by distillation.

Diethyl ether and toluene were dried by addition of sodium wire.

### 3.2 **Chromatographic Separation**

Thin Layer Chromatography (tlc)

Analytical tlc was performed using aluminium backed silica tlc plates (Merck 5554) developed in one of four solvent systems (unless otherwise stated ).

Solvent Systems;

A. 10% methanol in dichloromethane

B. 20% methanol in dichloromethane

C. 50% ethylacetate in dichloromethane

D. 5% methanol in dichloromethane

After developing, the results were viewed under uv light (254nm or 280nm) and were visualised with either iodine or anisaldehyde staining.

Anisaldehyde Staining

A solution containg 2% anisaldehyde, 2% acetic acid and 5% sulphuric acid in ethanol was sprayed onto developed plates which were then heated on a hot plate for 5 minutes. All ribose containing spots are turned either dark blue or dark green while areas without ribose remain pale. ( This operation must be carried out in a fume cupboard as the anisaldehyde reagent is extremely toxic ).

Flash Chromatography

Chromatographic separation was carried out using silica gel (Fluka silica 60 mesh 230 - 400) and slight positive pressure (3psi) of nitrogen.

High Performance Liquid Chromatography ( HPLC )

Oligonucleotides were analysed by HPLC using either a Gilson or Perkin- Elmer system and detected by uv absorbance at 264nm. Strong Anion Exchange and Reversed Phase separation techniques were employed.

Strong Anion Exchange HPLC was carried out with hypersyl and partisil columns using a two solvent system and an elution gradient of 0 - 70 % solvent B.

Solvent A. 4g $KH_2PO_4$ in $H_2O$/acetonitrile 600/400ml adjusted to pH 6.4 with KOH aqueous solution.

Solvent B. 78g $KH_2PO_4$ in $H_2O$/acetonitrile 600/400ml adjusted to pH 6.4 with KOH aqueous solution.

Reversed Phase HPLC was carried out using $C_8$ reversed phase columns ( Brownlee Aquapore, reference number RP200 ) with one of three elution systems.

1. For all non-labelled oligonucleotides.

Solvent A. 0.1M $NH_4OAc$ in $H_2O$

Solvent B. 0.1M $NH_4OAc$ in $H_2O$/acetonitrile 8/2 v/v

Elution was by means of a gradient from 0 - 70% solvent B over a period of 20 minutes.

2. For Dinitrophenyl labelled oligonucleotides.

Solvent A. 0.1M $NH_4OAc$ in $H_2O$.

Solvent B. 0.1M $NH_4OAc$ in $H_2O$/acetonitrile 6/4 v/v.

Elution was with a solvent gradient from 0 - 70 % solvent B over a period of 20 minutes.

## 3.3 **Analytical Techniques**

Melting Points

Melting points were obtained using a Kopfler Hot Stage apparatus.

Elemental Analysis

Microanalysis was carried out by Mrs E.M$^{c}$Dougall using a Perkin-Elmer 240 Elemental Analyser.

X-Ray Crystallography

X-Ray data was collected, solved and refined by Dr G.A.Leonard using a Stoe- Siemens AED-4 diffractometer.

## 3.4 **Spectroscopic Techniques**

Nuclear Magnetic Resonance Spectroscopy

$^{1}$H NMR spectra were carried out using a Brucker WP 200 SY spectrometer by Mr J.R.A.Millar and Miss H.Grant. Chemical shifts are quoted in parts per million against a tetramethylsilane standard.

$^{31}$P NMR were obtained using a Jeol JMN FX90Q spectrometer by Mr P Tan. The chemical shifts are quoted with reference to a phosphate standard.

Infra-Red Spectroscopy

Spectra were taken using a Perkin Elmer 781 spectrometer, with samples as nujol mulls between sodium chloride plates and were calibrated against a polystyrene standard (1603 $cm^{-1}$).

Mass Spectroscopy

All mass spectra were obtained using positive or negative Fast Atom Bombardment (FAB) ionisation techniques and were carried out on a Kratos MS 50 spectrometer by Mr A.Taylor.

Ultraviolet spectroscopy

Spectroscopy was carried out on a Perkin-Elmer Lamda 15 double beamed spectrometer.

Oligonucleotide Synthesis

Oligonucleotide synthesis was carried out on an Applied Biosystems (ABI) 380B oligonucleotide synthesiser using standard reagents and cycles unless otherwise stated. Cycles are included in Appendix A. All syntheses were of 0.2micromole scale

Snake Venom Digest

A sample of oligonucleotide( approximately 2 OD units) was dissolved in tris buffer pH 8.8 and to this was added 0.2mg of snake venom phosphodiesterase (Sigma ) and 5 microlitres alkaline phosphatase solution ( Sigma). This mixture was then incubated overnight at 37°C. Analysis was by reversed phase HPLC (elution system the same as for normal oligonucleotides).

4,4'-Dimethoxytrityl Analysis

The sample for analysis was dissolved in 60% hydrochloric acid in ethanol solution (25ml for the deprotection samples from a 0.2 micromole synthesis) and the absorbance at 495nm measured spectrophotmetrically. At 495 nm 1micromole of 4,4'-dimethoxytrityl cation gives a reading of approximately 71.7 Absorbance Units.

# Experimental

## Compound Index

(1) 5'-O-(4,4''-Dimethoxytrityl)thymidine

(2) 5'-O-(9-Fluorenylmethoxycarbonyl)thymidine

(3) Triethylammonium[5'-O-(9-fluorenylmethoxycarbonyl)-thymidine-3'-O-(2-chlorophenylphosphate)]

(4) 5'-O-(4,4''-Dimethoxytrityl)thymidine-3-O-succinate

(5) 5'-O-(4,4''-Dimethoxytrityl)thymidine

(6) 5'-O-(4,4''-Dimethoxytrityl)thymidine-3'-O-succinyl-glycine ethyl ester

(7) 5'-O-(4,4''-Dimethoxytrityl)thymidine-3'-O-succinyl-sarcosine ethyl ester

(8) 3'-O-Benzoylthymidine

(9) 5'-O-succinyl-3'-O-benzoylthymidine

(10) 3'-O-Benzoylthymidine-5'-O-succinyl-glycine ethyl ester

(11) 5'-O-(4,4''-Dimethoxytrityl)thymidine-3'-O-glutarate

(12) 5'-O-(4,4''-Dimethoxytritylthymidine-3'-O-glutaryl-glycine ethyl ester

(13) N-(9-Fluorenylmethoxycarbonyl)sarcosine

(14) Sarcosyl thymidine resin

(15) Sarcosyl adenosine resin

(16) Phthaloyl thymidine resin

(17) Fluorenyl-9-yl methanol

(18) Fluorenyl-9-ylmethylene chloroformate

(19) 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine

(20) 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine-3'-O-N,N-diisopropylmeth yl-phosphoramidite

(22) 5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-isobutyryl-2'-deoxyguanosine

(23) 5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylcyanoethylphosphoramidite

(24) 5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylmethyl phosphoramidite

(25) 4-N-Adamantoylcarbonyl-2'-deoxycytidine

(26) 2-N-(4,4''-Dimethoxytrityl)-2'-deoxyguanosine

(27) 4-N-(4,4''-Dimethoxytrityl)-2'-deoxycytidine

(28) 6-N-(4,4''-Dimethoxytrityl)-2'-deoxyadenosine

(29) 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4''-dimethoxytrityl)-2'-deoxyadenosine

(30) 5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrityl)-2'-deoxyguanosine

(31) 5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine

(32) 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4''-dimethoxytrityl)-2'-deoxyadenosine-3'-O-N,N-diisopropylmethyl phosphoramidite

(33) 5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrityl)-2'-deoxygunosine-3'-O-N,N-diisopropylmethyl phosphoramidite

(34) 5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine-3'-O-N,N-diisopropylmethyl phosphoramidite

(35) 4-N-benzoylcytidine

(36) 3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine

(37) 2'-O-(4-methoxytetrahydropyran-4-yl)-3',5'-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine

(38) 2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine

(39) 5'-O-(9-Fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine

(40) 5'-O-(9-Fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine-3'-N,N-diisopropylphosphoramidite

(41) Methoxyethoxymethyl triethylammonium chloride

(42) Methoxyethoxmethyl-3',5'-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine

(43) 4-N-Phthaloylcytidine

(44) 6-N-Phthaloyladenosine

(45) 3',5'-(Tetraisopropyldisiloxane-1,3-diyl)-6-N-phthaloyladenosine

(46) Methoxyethoxymethyl-6-N-phthaloyl-3',5'-(tetraisopropyldisiloxane-1,3-diyl)-adenosine

(47) Di-*n* - Butylformamide dimethyl acetal

(48) 3',5'-(Tetraisopropyldisiloxane-1,3-diyl)adenosine

(49) 6-N-Di-n-Butylformamidine-3',5'-(tetraisopropyldisiloxane-1,3-diyl)adenosine

(50) 5'-O-(4,4''-Dimethoxytrityl)-2'-deoxycytidine

(51) Product from the reaction of ethylenediamine with 2,4-dinitrofluorobenzene

(52) 2'-Deoxycytidine refluxed with ethylenediamine

(53) Reaction of (**52**) with dinitrofluorobenzene

(54) Reaction of (**52**) with FMOC-succinimide

(55) N-(9-Fluorenylmethoxycarbonyl)-6-aminohexan-1-ol

(56) N-(9-Fluorenylmethoxycarbonyl)-6-aminohexan-1-ol-1-N,N-diisopropylcyanoethylphosphoramidite

(57) N-(2,4-Dinitrophenyl)-6-aminohexan-1-ol

(58) 1-O-(4,4''-Dimethoxytrityl)-6-N-(2,4-Dinitrophenyl)-6-aminohexan-1-ol

(59) N-(2,4-dinitrophenyl)-6-aminohexan-1-ol-1-O-N,N-diisopropylcyanoethylphosphoramidite

(60) 3-N-dinitrophenyl-3-amino-1,2-propanediol

(61) 1-O-(4,4'-dimethoxytrityl)-3-N-(2,4-dinitrophenyl)-3-amino-1,2-propanediol

*5'-O-(4,4''-Dimethoxytrityl)-thymidine* (**1**).-Thymidine(4.84g,20mmol), dried by coevaporation from dry pyridine, was dissolved in dry pyridine(150ml) and dimethylaminopyridine (DMAP) (122mg,0.05 equivalents) , triethylamine(3.8ml,1.4 equivalents) and dimethoxytrityl chloride (8.124g,1.2 equivalents ) were added. The solution was stirred for 2 hours and the reaction followed by tlc (10% MeOH , 2% TEA, in dichloromethane). 4,4'-Dimethoxytrityl chloride (2.2g,0.2 eq) and triethylamine(1.4ml,0.5eq) were then added and after a further hour the reaction mix was poured into an equal volume of water. The product was extracted with ether (2x500ml) and the organic phase evaporated to dryness. Purification by flash chromatography ( 2% MeOH, 2% triethylamine in dichloromethane) gave 5'-*O-(*4,4''-*dimethoxytrityl)-thymidine* (**1**) (10.89g, 99%), m.p. 118-120°C (lit. value (Aldrich) 117-121°C).

*5'-O-( 9-Fluorenylmethoxycarbonyl)-thymidine* (**2**).-To dry thymidine(1g,) in anhydrous pyridine(15ml) was added 9-fluorenylmethoxycarbonyl chloride ( 1.2 eqs) .The reaction was protected from moisture with a silica gel drying tube and stirred for 2 hours at room temperature.When tlc (5:1 EtOAc:MeOH) indicated that the reaction was complete the solution was poured into water(200ml) and extracted into chloroform(5x30ml ).The organic phase was dried over sodium sulphate and evaporated to dryness. Crystallisation from dichloromethane gave *5'-O-(9-fluorenylmethoxycarbonyl)-thymidine* (**2**) (0.96g, 50% ), m.p. 188-9°C (lit.,[64] 190°C); $\lambda_{max}$ (MeOH) 265nm ($\varepsilon$ 10,000 $dm^3mol^{-1}cm^{-1}$); m/z 487 (30%), 465( $M+H^+$, 100%).

5'-(9-Fluorenylmethoxycarbonyl)-thymidine (**2**) stability in organic solvents (t.l.c. system A).

10% DBU in acetonitrile ---- complete deprotection in 4 mins

10% DBU in acetonitrile + 1 eq acetic acid-- complete deprotection in under 40 mins .

10% DBU in acetonitrile +2equivalents acetic acid ---- incomplete deprotection.

3'-O-Benzoylthymidine was not affected by 10%DBU in acetonitrile.

5'-( 9-Fluorenylmethoxycarbonyl)-thymidine was stable in ; ethanol, methanol, acetonitrile, chloroform, pyridine, dichloromethane, 1.2-dichloroethane (t.l.c. system A).

5'-(9-Fluorenylmethoxycarbonyl)-thymidine (**2**) was stable in freshly distilled DMF, but

decomposed when dissolved in SLR grade DMF (t.l.c. system A).

*Triethylammonium[- 5'-O-( 9-fluorenylmethoxycarbonyl)-thymidine-- 3'-O-( 2-chlorophenyl phosphate)].* (**3**).- Chlorophenylphosphorodichloridate (0.2ml) was added to a solution of 1,2,4-triazole (0.23g,3.29mmol) in dry triethylamine (0,4 ml, 2.39mmol).After 30 minutes the white solid triethylamine hydrochloride was filtered off and the clear solution added to dry 5'-(9-fluorenylmethoxycarbonyl)thymidine(0.4g,0.86mmol) and left for 5 minutes. The product was extracted with dichloromethane(3x3ml), dried over sodium sulphate, and evaporated to dryness.

The product was precipitated from dichloromethane into dry n-pentane (with vigorous stirring), to give *Triethylammonium[5'-O-( 9-fluorenylmethoxycarbonyl)-thymidine- 3'-O-( 2-chlorophenylphosphate)]* (**3**) (0.357g, 64%); m/z 735 [M-($H^+$), 80%].

*Stability of succinyl linkage to resin to 10% DBU solution on pyridine.*- High loading resin 150x10(-6) mol/g was tested.

Two samples(each 10mg) were prepared; to the first was added 10% DBU in dry dichloromethane (v/v,1ml)and the second was soaked in dry dichloromethane(1ml) as a control. After 40 minutes the resin was removed by filtration and the filtrates made up to 25ml with 60% hydrochloric acid/ ethanol.The DBU solution gave a deep orange colouration characteristic of trityl cation. Cleavage of the first nucleotide from the resin had occurred. The solution from the control remained clear.

*5'-O-( 4,4"-Dimethoxytrityl)thymidine -3'-O- succinate* (**4**).- To dry 5'-O-(4,4"-dimethoxytrityl)thymidine ( 0.17g,0.3mmol ), dissolved in anhydrous pyridine, was added succinic-anhydride ( 0.03g,0.28mmol) and DMAP ( 0.02g,0.2mmol).The mixture was stirred at room temperature for 24 hours and the reaction was monitored by tlc (system A) against a standard sample of the succinate. When the reaction was complete by tlc(system A), dichloromethane(20ml) was added and the mixture washed with water (2x5ml).The organic portion was dried over sodium sulphate, evaporated to a small volume and precipitated into dry n-hexane to give 5'-*O-( 4,4"-dimethoxytrityl)thymidine-3'-O-succinate* (**4**). (0.09g, 48%), [Found: C, 67.2; H, 5.5; N, 4.35; O, 24.9%; M(mass spectrum), 643.22918. $C_{36}H_{35}N_2O_{10}$ calc. C, 67.2; H, 5.5; N, 4.35; O, 24.9%; M, 643.22915]; m/z 643 [M-(H+), 80%], 543 (DMTrT, 100), 199, 99.

*Reaction of 5'-O-( 4,4" -Dimethoxytrityl)thymidine -3'-O- succinate (4), with glycine ethyl ester.*- 5'-O-(4,4"Dimethoxytrityl)thymidine-3'-O-succinate (0.2g,0.3mmol) was dissolved in a minimum volume of dichloromethane and DCCI (0.04g,0.15mmol) was added. This mixture was filtered into neutralised glycine methyl ester(0.04g/0.04ml triethylamine )

The reaction was followed by t.l.c(system A), Rf starting material = 0.2, Rf product = 0.45, but did not go to completion,some starting material remained.The organic phase was washed with citric acid solution(15ml of 5%) and water (10ml) and dried over sodium sulphate and evaporated to dryness to give only product (**5**) (66mg, 30%); m/z 543 ( DMTr T, 100%).

5'-*O-( 4,4"-dimethoxytrityl)thymidine- 3'-O-succinyl-glycine ethyl ester* (**6**).--5"-O-(4,4"-dimethoxytrityl)thymidine-3'-O-succinate (0.1266g,0.20mmol) and hydroxybenzotriazole (0.02g,0.15mmol) were dissolved in dioxan/pyridine (0.5/0.3 ml) and DCC (0.07g,0.34mmol) was added. The mixture was stirred at room temperature for 2.5 hrs until all the starting material had been consumed.The white precipitate of N,N dicyclohexylurea (DCU)) was removed by filtration and the filtrate was added to the glycine ethyl ester (0.030g,0.26mmol), previously liberated from its hydrochloride with diisopropylamine. This mixture was stirred at room temperature overnight. After addition of dichloromethane(2ml) the organic phase was washed with citric acid (5%,10ml),

water (10ml) , sodium bicarbonate solution (50%,10ml) and water (10ml),dried over sodium sulphate,evaporated to a small volume and precipitated into hexane to give solid *5'-O-( 4,4''-dimethoxytrityl)thymidine- 3'-O-succinyl-glycine -ethylester* (**6**).(60mg, 40%) (Found C, 64.9; H, 6.8; N, 6.02. $C_{39}H_{43}N_3O_{11}$ requires C, 64.9; H, 6.8; N, 6.0%); $\lambda_{max}$ (MeOH) 207nm (Ɛ 41,044 $dm^3mol^{-1}cm^{-1}$), 223nm (14,640), 266nm (6,303); $\delta_H$ (200MHz; $CDCl_3$) 1.7 ( 3H, s, methyl of T) 2.4-2.75 (7H, m, $C_2$'+succinyl Hs), 2.9(2H, d, $CH_2$), 3.5 (2H, s, $C_5$' H), 3.8 (6H, s, methoxy of DMTr), 4.0 (2H, d, $C_4$'), 4.2 (2H, q, $CH_2$ ester), 5.5 (1H, d, $C_3$'), 6.1 (1H, t, $C_1$' ), 6.4 (1H, t, NH), 6.8 (4H, d, DMTr), 7.1-7.4 (9H, m, DMTr), 7.6 (1H, s, T 6H), 8.2 (1H, s, N(3)H of T).

*5'-O-(4,4''dimethoxytrityl)thymidine-3'-O-succinyl sarcosine ethyl ester.* (**7**).-- 5''-O-(4,4''dimethoxytrityl)thymidine-3'-O-succinate (0.1266g,0.20mmol) and hydroxybenzotriazole (0.02g,0.15mmol) were dissolved in dioxan/pyridine (0.5/0.3 ml) and DCC (0.07g,0.34mmol) was added. The mixture was stirred at room temperature for 2.5 hrs until all the starting material had been consumed.The white precipitate of N,N dicyclohexylurea (DCU)) was removed by filtration and the filtrate was added to sarcosyl ethyl ester (0.0373g,0.26mmol), previously liberated from its hydrochloride with diisopropylamine. This mixture was stirred at room temperature overnight. After addition of dichloromethane(2ml) the organic phase was washed with citric acid (5%,10ml), water (10ml) , sodium bicarbonate solution (50%,10ml) and water (10ml),dried over sodium sulphate, evaporated to a small volume and precipitated into hexane to give solid *5'-O-(4,4''dimethoxytrityl)thymidine-3'-O-succinyl sarcosine ethyl ester.* (**7**) (62mg, 40%) [ Found: C, 64.7; H,6.0; N, 5.7; O, 24.2%; M (mass spectrum), 742.29759. $C_{40}H_{44}N_3O_{11}$ requires: C, 64.7; H, 6.0; N, 5.7; O, 24.2%; M, 742.29755.]; $\lambda_{max}$ (MeOH) 207nm ( Ɛ 49,954 $dm^3\ mol^{-1}\ cm^{-1}$), 233 (20,817), 266 (9,842);

$\delta_H$ (200MHz, $CDCl_3$) 1.3 (6H, m, Me/T+ester), 1.7 (3H, s, Me/sarcosine), 2.7 (2H, s, $CH_2$/ sarcosine), 3.0 (1H, d, $C_3$'), 3.1 (1H, s, $C_4$'), 3.4 (2H, d, $C_5$'), 3.75 (3H, s, methoxy), 4.0-4.25 (4H, m, succinyl $CH_2$), 5.5 (1H, s, water), 6.4 (1H, t, $C_1$'), 6.8 (4H, m, DMTr), 7.2-7.5 (9H, m, DMTr), 7.6 (1H, s, 6H/T), 8.1 (1H, s, N-H of T).

*Stability of glycine and sarcosine model compounds to 10% DBU solution in dichloromethane.-* Model compound(10mg) was dissolved in dichloromethane(1ml) and 10% DBU in dichloromethane(1ml) was added. The stability of the sample was monitored by tlc (system D). The glycine compound (**6**) was immediately decomposed to give a product with the same Rf value as 5'-O-(4,4"dimethoxytrityl)thymidine.

The sarcosine compound (**7**) compound was stable to 10% DBU solution for at least 24 hours.

*10% DBU solution in methanol.-* Both compounds (**6**) and (**7**) were broken down within 30 minutes when dissolved in this solution.

*Triethylamine.-* Both compounds (**6**) and (**7**) showed no change ( by tlc ) when treated with excess triethylamine for 1 hour.

3'-*O-Benzoylthymidine* (**8**).-To a stirred solution of dry 5'DMTrT(0.436g,0.8mmol) and DMAP(0.02g) in dry pyridine(1.2ml) was added benzoic anhydride(0.27g,1.5equivalents).After 4 hours the mixture was poured into water(40ml), extracted into dichloromethane(3x10ml) and evaporated to dryness.The residue was then dissolved in 80% acetic acid(20ml), stirred and warmed (35°C),until detritylation was complete (monitored by system A). Acetic acid was removed by evaporation under reduced pressure and azeotroping with toluene to give solid 3'-*O-Benzoylthymidine* (**8**) (100mg, 50%); $\lambda_{max}$ 201nm ( $\varepsilon$ 15,556 $dm^3\ mol^{-1}\ cm^{-1}$), 228 (13,049), 265 (8,874).

5'-*O-succinyl-* 3'-*O-benzoylthymidine* (**9**).-3'-O-Benzoylthymidine( 68mg ) was dissolved in anhydrous pyridine(1ml)in the presence of DMAP(catalytic amount).To this solution succinic anhydride (2equivalents) was added in portions over 20 minutes.The solution was stirred followed by stirring at room temperature overnight, diluted with dichloromethane(15ml) and washed with 10%citric acid(2ml) and ice cold water (5ml).The organic phase was dried over sodium sulphate and evaporated to dryness.Crystallisation from diethylether gave 5'-*O-succinyl-*3'-*O-benzoylthymidine* (**9**) (95mg, 74%) (Found C, 56.8; H, 5.0; N, 6.3%. $C_{21}H_{22}N_2O_9$ requires C, 56.5; H, 5.0; N, 6.3% ).

3'-*O-Benzoylthymidine-* 5'-*O-succinylglycine ethyl ester* (**10**).-Hydroxybenzotriazole (0.02g,0.14mmol)and the 5'-O-succinate (100mg, 0.14mmol) were dissolved in dioxan/pyridine (0.5ml/0.03ml)and DCC (0.07g,0.34mmol) was added. This mixture was stirred at room temperature 2.5 hours until all the starting material had been consumed(monitored by system A).The mixture was then filtered into a solution of glycine ethyl ester (previously liberated from its hydrochloride with triethylamine) and stirred at room temperature overnight, after which it was diluted with dichloromethane (10ml) and washed with 5% citric acid solution (2ml),water (2ml), 10%sodium bicarbonate solution (2ml) and water (2ml). The organic phase was dried over sodium sulphate and evaporated to dryness. Precipitation from dichloromethane by the addition of n-hexane yielded solid 3'-*O-Benzoylthymidine-* 5'-*O-succinylglycine ethyl ester* (**10**) (35mg, 48%)mp 124-7°C (Found C, 55.6; H, 5.5; N, 8.2%. $C_{25}H_{29}N_3O_{10}$ requires C, 55.4; H, 5.5; N, 7.9%).

5'-*O-(* 4,4"-*Dimethoxytrityl)-thymidine-* 3'-*O-glutarate* (**11**).--Dry DMTrthymidine (0.172g) was dissolved in anhydrous pyridine and protected from moisture with a silica gel guard tube. Glutaric anhydride(2.5 equivalents) was added and the reaction mixture was stirred at room temperature overnight and monitored by tlc (system A) (product Rf =0.25). After removing pyridine at reduced pressure the white oil was redissolved in dichloromethane(20ml) and washed with water (3x5ml). The organic layer was then dried over sodium sulphate and the solvent removed on a rotary evaporator. The product was precipitated into dry hexane from a small volume dichloromethane to give solid 5'-*O-(*4,4"-*Dimethoxytrityl)-thymidine-*3'-*O-glutarate* (**11**) (0.102g, 50%) [Found: C, 65.8; H, 5.7; N, 4,3; O, 24.3%; M (mass spectrum), 657.24485. $C_{36}H_{37}N_2O_{10}$requires C, 65.8; H, 5.7; N, 4.3; O, 24.3%; M, 657.24480]; $\lambda_{max}$ 206nm ($\varepsilon$ 45,145 $dm^3\ mol^{-1}\ cm^{-1}$), 232 (17,772), 266 (8,591); m/z 657 [ M-($H^+$), 75%], 543 (DMTr ), 125 (30%)

*5'-O-(4,4"-Dimethoxytrityl)thymidine--3'-O-glutarylglycine ethyl ester.*(**12**).--To the dry glutarate (**11**) (0.2mmol ) and hydroxybenzotriazole (0.03g,1equivalent) in dioxan/pyridine (0.5/0.3ml) DCC (2.5 equivalents, ) was added and the mixture stirred for 2.5 hours .The DCU produced was

filtered off and the active ester added to glycine ethyl ester (liberated with diisopropylethylamine (1equivalent) ). This solution was then stirred overnight after which time dichloromethane(10ml) was added and the solution washed with water(2ml), citric acid solution (10%,2ml), water(2ml) and sodium bicarbonate solution (50%, 2ml). The organic phase was dried over sodium sulphate, evaporated to dryness and the product precipitated from dichloromethane into n-hexane to give *5'-O-( 4,4''-Dimethoxytrityl)thymidine- -3'-O-glutarylglycine ethyl ester.* (**12**) (49%) [Found: C,64.6; H, 6.1; N, 5.6; O, 23.7%; M(mass spectrum), 742.29756. $C_{40}H_{44}N_3O_{11}$ requires C, 64.6; H, 6.1; N, 5.6; O, 23.7%; M, 742.29756 ]; $\lambda_{max}$ (MeOH), 204nm ( $\varepsilon$ 67,147 $dm^3 mol^{-1} cm^{-1}$ ), 233 (21,166), 266 (9,598); m/z 778 (30%), 742 [M+($H^+$), 70), 543.

*Stability.*- Small samples of the model compound (**12**) were dissolved in the following solvents and their stability monitored by tlc (system A).

1/. 10% DBU in dichloromethane/chloroform led to 50% cleavage in 2hours

2/. 5% piperidine in acetonitrile led to 50% cleavage in approximately 48hours

3/.morpholine:acetonitrile 30:70: compond (**12**) was stable for 48 hours

4/.triethylamine:acetonitrile 30:70: compound (**12**) was stable for 48 hours

*N-( 9-fluorenylmethoxycarbonyl)-- sarcosine* (**13**). --To sarcosine ( 1.9g , 21mmol) dissolved in sodium carbonate ( 10%sol. 18ml) was added dimethylacetimide(DMA) ( 18ml). The solution was cooled to 0°C and a solution of 9-fluorenylmethoxycarbonyl chloride dissolved in DMA (4g in 12ml) was added. The reaction was stirred for 15 mins while it warmed to room temperature. Water (240ml ) was then added to the mixture and the product was extracted with diethyl ether (40ml) followed by ethyl acetate (2x40ml). The aqueous phase was then acidified to pH 1.5 with concentrated hydrochloric acid and further product was extracted with diethyl ether ( 6x30ml ). The combined organic phase was dried and the solvent removed under reduced pressure. Crystallisation from ether gave *N-( 9-fluorenylmethoxycarbonyl)-- sarcosine* (**13**) (2.6g, 41%) mp 119-120°C standard value (Cambridge Research Biochemicals 121-123°C.

*Resin functionalisation.*- 1g of long chain alkylamine controlled pore glass resin with 20 micromol/g loading was functionalised with a sarcosine spacer unit and a succinyl thymidine first nucleoside.

The resin of 500 Angstrom pore size was first dried over phosphorus pentoxide.

Stages in functionalisation;

1. Wash with 10% vv diisopropylamine in DMF(3x20ml).

2.Wash with DMF ( 3x20ml)

*Activation of 9-fluorenylmethoxycarbonyl-sarcosine.*- To 9-fluorenylmethoxycarbonyl-sarcosine (0.0622g , 10 equivalents relative to the resin loading ) dissolved in the minimum volume of DMF add DCC (0.02069g , 5 equivalents) . Stir for 20 mins and then evaporate to dryness and redissolve in dichloromethane(15ml). Filter this mixture into the resin functionalisation vessel.

3. Add activated 9-fluorenylmethoxycarbonyl-sarcosine and allow to react 90 mins.

4. Wash with DMF (3x20ml).

5. Add a second batch of activated 9-fluorenylmethoxycarbonyl -sarcosine and allow to react for 90 mins.

6. Wash with DMF (3x20ml)

7. Kaiser test[2] for free amine to check that all available amine sites have in fact reacted with the protected sarcosine.

8. Wash with piperidine/DMF (2/8 v/v, 20ml) 10minutes

9. Wash with DMF (3x20ml)

10. Wash with piperidine/DMF (2/8v/v,20ml) 10 minutes.

11. Wash with DMF (3x20ml)

12. Wash with dichloromethane(4x20ml)

13. Kaiser test to check that amine sites are deprotected

14. Add activated nucleoside succinate and allow to react for 5.5 hours.

15. Wash with dichloromethane(5x20ml)

16. Wash with diethyl ether (3x20ml)

17. Couple nucleoside succinate to the resin

*Symmetric anhydride of nucleoside succinate.*- Dissolve the protected nucleoside succinate (10 equivalents over resin loading) in dry dichloromethane and add DCC (5 equivalents over resin loading). After 20 minutes filter the mixture into the resin functionalisation vessel and the coupling reaction is then left for 5 hours under nitrogen at room temperature.

After the nucleoside coupling reaction the loading of the resin is determined as follows:

A known amount of resin is suspended in HCl/EtOH (60/40) (25ml) and, after allowing 5 mins for complete deprotection a visible spectrum can be taken at 498nm. From this it is possible to calculate the amount of protected nucleoside attached to the resin (1 micromol of dimethoxytrityl cation has an absorbance of 70 AU).

Unreacted sites within the resin structure can then be 'capped off ' by acetylation to prevent formation of uncleavable phosphate linked nucleotides.

17. Acylation. Set aside the resin in acetic-anhydride/pyridine (2/8v/v,20ml) solution for 2.5 hours.

18. Wash with dichloromethane(4x20ml)

19. repeat '17'.

20. repeat '18'

21. wash with diethyl ether (3x20ml)

*Sarcosyl thymidine resin.* (**14**).- Prepared by general method above gave support of loading 18.828 micromol/g.

*Sarcosyl deoxyadenosine Resin* (**15**).-same method after 2 couplings of nucleoside succinate gave 25micromol/g loading.

*Stability of sarcosyl resin to non-nucleophilic bases.*- A sample of sarcosyl thymidine resin was soaked in 10% DBU/ pyridine solution for 18 hours and the resin was removed by filtration,the solution assayed by spectrophotmetry at 495nm.

To 25ml HCl/EtOH 60/40 was added the solution of DBU which had been used to treat 11.6mg of resin .

Reading for 1ml path volume =.002 A.U.

Total absorbance of sample = .002 x 25 = .050 A.U.

Amount of dimethoxytrityl cation in sample = 0.05/71.7 = 0.0007micromol

Resin loading = (0.0007/11.6) x 1000 = 0.06micromol/g

Loss rate of 0.02% per hour

*Stability of sarcosyl resin to DBU in acetonitrile.*-Sarcosyl thymidine resin (7.2mg ) was soaked in 10% DBU in acetonitrile overnight.The resin was removed by filtration and the DBU/acetonitrile solution was assayed for DMTr to see if any of the first base had been cleaved from the resin.

Dilution to 10ml with 60% concentrated hydrochloric acid in ethanol

1cm path length

absorbance at 495nm = 0.075A.U.

= 0.075x 10 / 71.7 micro mol

= 0.4% per hour cleaved

*Phthaloyl thymidine resin.* (**16**).-Prepared as follows:

1/. Resin(0.5g) soaked in 10% diisopropylethylamine in dichloromethane(10ml) for 20 minutes.

2/. Wash with DMF(2x10ml)

3/. Add 10 equivalents of activated DMTr T phthallate

( activation by 5 equivalents of DCC in dichloromethane)

4/. Allow to stand overnight ( under nitrogen)

The resulting resin had loading of 11.02 micromol /g.

*Stability to DBU.*- A sample of resin(20.5mg) was treated with 10% DBU in dichloromethane for 10 minutes and then both the resin and the washings were assayed for dimethoxytrityl content by treatment with acid and spectrophotometric measurement.

20.5mg (11.06 micromol/g)

10.6micromol/g cleaved

0.99micromol/g remaining

= 91% cleaved in 10 minutes

$t_{1/2}$ <5minutes

*Stability to aqueous ammonia.*- Samples of resin were treated with concentrated ammonia and then after washing assayed for loading by the acid/spectrophotometric method.

Phthaloyl resin was 85% cleaved in 23 minutes

CPG dC commercial resin 42% cleaved in 23 minutes

*Fluorenyl-9-yl methanol* (**17**).- Sodium-hydride (80% dispersion in mineral oil ) ( 2.1638g ) was suspended in sodium dried diethyl-ether (35ml) and fluorene ( 2.7002g ) and ethylformate (3ml) were added. The mixture was refluxed for 10 hours and then cooled on ice . Ice chips were added to the mixture until gas evolution ceased. Water(150ml) was then added, the ether layer was discarded and the aqueous layer was washed with light petrol (b.p.40-60oC ) (40ml) and filtered. The aqueous phase was cooled on ice,acidified with acetic acid (15ml) and the resulting oil extracted with dichloromethane,dried over sodium sulphate, and evaporated to dryness under reduced pressure. The oil was dissolved in methanol (30ml) and sodium borohydride (0.99g) was added. This mixture was stirred at room temperature for 2 hours , after which time water(70ml) and acetic acid ( 5ml) were added. After stirring for a further 1.5 hours the white solid was filtered off and recrystallised from light petrol (b.p. 80-100°C ) to give *fluorenyl-9-yl methanol* (**17**) (60%), m.p. 98-100°C (lit. value[114] 100°C ).

*Fluorenyl-9-ylmethylene chloroformate* (**18**). [114]- Phosgene in toluene (12%) (12.5ml) was cooled to 0°C and fluorenyl-9-yl methanol (2.4g) was added gradually. After addition of dichloromethane (14ml) the mixture was stirred for 5 hours at ice bath temperature. Solvent and excess phosgene were then evaporated off and the product recrystallised from diethyl ether/petroleum ether 40-60 to give *fluorenyl-9-ylmethylene chloroformate* (**18**) (15%), m.p. 62-64°C (lit. value[114] 61.5-63°C); $\nu_{max}$ 3200 $cm^{-1}$, 1790, 1150.

*5'-O-(9-fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine* (**19**).- 6-N-Benzoyl-2'-deoxyadenosine (1.2g,3.4mmol), coevaporated from pyridine, was dissolved in anhydrous pyridine(30ml) and to this solution FMOC-Cl (1.0g ,1.2 equivalents) was added. The reaction mixture was protected from moisture with a calcium chloride guard tube and light by aluminium foil and stirred at room temperature for 3 hours.

The reaction was followed by tlc(system B) and when complete the reaction mixture was poured into water (120ml) and extracted with dichloromethane(3x35ml).This organic phase was then dried over sodium sulphate and evaporated to dryness.The product was solidified by trituration with diethyl ether.

Flash column chromatography(30ml methanol in 250ml dichloromethane) and evaporation to dryness yielded the stable foam*5'-O-(9-fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine* (**19**) (0.5g, 25%); [Found: C, 66.5; H, 4.9; N, 12.1; O, 16.6%; M (mass spectrum), 578.20394. $C_{32}H_{28}N_5O_6$ Calc. C, 66.5; H, 4.9; N, 12.1; O, 16.6%; M, 578.20395]; $\lambda_{max}$ 209nm ( $\varepsilon$ 47,109 $dm^3$ $mol^{-1}$ $cm^{-1}$ ), 265 (24,470); $\delta_H$(200MHz, $CDCl_3$ ), 2.1 (1H, broad, -OH), 2.5-2.8 (2H, d of m, $C_2'$), 3.45( 1H, s,), 4.2-4.5 (7H, m, C5',C4',$CH_2$Fmoc,CHFmoc), 3.9 ( 1H, h, water), 4.7 ( 1H, m, $C_3'$), 5.25 ( 1H, s, water), 6.5 ( 1H, t, $C_1'$), 7.2-7.8 ( 15H, m, Ar/Fmoc,Bz,$CHCl_3$,NH), 7.9-8.0( 2H, m, Bz), 8.25 ( 1H, s, Adenosine $C_8$), 8.75 ( 1H, s, Adenosine); m/z 686 (M+thioglycerol, 70%), 600 (40), 578 [ M+($H^+$), 60).

*5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine-3'-O-N,N-diisopropylmethylphosphoramidite* (**20**).- 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine ( 0.5g,0.9mmol), dried in vacuo for 3 days, was dissolved in dry dichloromethane (12ml) and the reaction vessel flushed with nitrogen. N,N-Diisopropyl-ethylamine (0.9ml,2 equivalents ) and methoxy-N,N-diisopropylchloro-phosphoramidite (0.2ml,1.2equivalents) were added. The reaction was then stirred under nitrogen for 15 mins, monitored by tlc(system C) Rf product = 0.55, Rf SM = 0. When the reaction was complete by tlc, anhydrous methanol ( 0.01ml) and ethyl acetate (21.4ml) were added and this solution washed with 10% sodium carbonate

solution(2x15ml) and brine (2x15ml). The organic phase was dried over sodium sulphate, evaporated to dryness and precipitated from ethyl acetate ( 35% w/v solution of product in ethyl acetate) into dry hexane to give solid *5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-benzoyl-2'-deoxyadenosine-3'-O-N,N-diisopropylmethylphosphoramidite* (**20**) (479mg, 72% ); $^{31}$P 149.9 (d); mz 793, 771, 739 [M+(H$^+$), 60%).

*5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-isobutyryl-2'-deoxyguanosine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**22**).- 5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-isobutyryl-2'-deoxyguanosine (0.5g,0.9mmol) dried in vacuo ( oil pump ) for 3 days was dissolved in dry dichloromethane(10ml) and the reaction vessel flushed with oxygen free nitrogen. N,N-Diisopropylethylamine(0.9ml, 2 equivalents ) was added and methoxy-N,N-diisopropylchlorophosphoramidite (0.2ml,1.2equivalents) was added.The reaction was stirred under nitrogen for 15 minutes,monitored by tlc(system C) .Rf product = 0.3-0.4 ,Rf SM = 0

When the reaction was complete by tlc (system C), anhydrous methanol(0.01ml) and ethyl acetate (21.4ml) were added This solution was washed with 10% sodium carbonate solution(2x15ml) and brine (2x15ml). The organic phase was dried over sodium sulphate, evaporated to dryness and precipitated from ethylacetate into dry hexane gave solid *5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-isobutyryl-2'-deoxyguanosine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**22**) (487mg, 75%); $^{31}$P 149.6 (d).

*5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylcyanoethylphosphoramidite* (**23**).- 5'-O-(9-Fluorenylmethoxycarbonyl)thymidine (0.767g,1.7mmol) was dissolved in dry THF (4ml) and to this diisopropylethylamine (1 equivalent ) and N,N-diisopropylcyanoethyl-chlorophosphoramidite( 2equivalents ) were added . This mixture was stirred under nitrogen for 60 minutes and monitored by tlc (system C,Rf product = 0.45 and 0.55 two isomers separate). When the reaction was complete, ethyl acetate presaturated with nitrogen, (40ml) was added. The organic phase was then washed with sodium carbonate solution ( 10 % 2x12ml) and brine (2x12ml), dried over sodium sulphate and evaporated to a dry foam. The product was precipitated

by addition of a dry toluene solution to a large volume of n-hexane giving *5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylcyanoethyl-phosphoramidite* (**23**) (0.6713g, 60%); $^{31}$PMR 149.9, 149.4.

*5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**24**).-5'-O-(9-Fluorenylmethoxycarbonyl)thymidine (0.8g), dried by coevaporation from dry pyridine, was dissolved in dry dichloromethane (12ml) and the reaction vessel flushed with nitrogen. N,N-Diisopropyl-ethylamine (0.95ml,2 equivalents ) and methoxy-N,N-diisopropylchloro-phosphoramidite (0.35ml,1.2equivalents) were added. The reaction was then stirred under nitrogen for 15 mins, monitored by tlc (system C) Rf product = 0.47, Rf SM = 0. When the reaction was complete by tlc anhydrous methanol ( 0.01ml) and ethyl acetate (21.4ml) were added and this solution washed with 10% sodium carbonate solution(2x15ml) and brine (2x15ml). The organic phase was dried over sodium sulphate and evaporated to dryness and precipitated from ethyl acetate into dry hexane to give solid *5'-O-(9-Fluorenylmethoxycarbonyl)thymidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**24**) (563mg, 60% ); $^{31}$P 149.8, 150.2 (d); m/z 793, 771, 739 [M+(H$^+$), 60%).

*4-N-Adamantyloxycarbonyl-2'-deoxycytidine* (**25**).-2'-Deoxycytidine ( 132mg,0.6mmol) was suspended in dry pyridine(3ml) and chlorotrimethylsilane (10 equivalents ) was added. After 40 minutes adamantyl fluoroformate ( 200mg, 2equivalents) was added and the reaction mixture, protected from moisture with a silica gel drying tube, was stirred overnight.The product, (tlc(system A) Rf product = 0.2), was isolated by adding water/pyridine(1/1,2ml) to the mixture, followed, after 30 minutes by more water(20ml). The aqueous phase was extracted with chloroform (5x4ml), dried over sodium sulphate and evaporated to dryness.The product was recovered by addition of a dichloromethane solution to a large volume of n-hexane to give solid *4-N-Adamantyloxycarbonyl-2'-deoxycytidine* (**25**) (90.7mg, 45%); $\lambda_{max}$ 211nm ( $\varepsilon$ 15,507 dm$^3$ mol$^{-1}$ cm$^{-1}$ ), 242 (12,513), 292 (6,309); $\delta_H$ (200 MHz, $CDCl_3$ ), 1.6 ( 6H, s, Adoc), 1.9-2.3 ( 11H, m, Adoc+$C_2$'), 3.6 (2H, m, $C_5$'), 3.8 ( 1H, m,8, $C_4$'), 4.25 ( 1H, m, $C_3$'), 5.0-5.3 ( broad, water/OH),

6.1 ( 1H, t, $C_1$'), 7.0 ( 1H, d, $C_5$), 8.25( 1H, d,$C_6$), 10.3 ( 1H, h, NH); m/z 476 (40%), 404 [ M-($H^+$), 100], 136 (100).

*Deprotection.*- The group was stable to TCA ( 3% in dichloromethane) and acetic acid ( 80%) but was removed by 60% HCl in ethanol in under 10 minutes monitored by t.l.c.

*2-N-( 4,4'-Dimethoxytrityl)-2'-deoxyguanosine* (**26**).- 2'-Deoxyguanosine( 0.57g,2.1mmol) was suspended in dry pyridine (8ml) and trimethylsilyl chloride (2.7ml,5equivalents) was added. This mixture was stoppered and stirred for 30 minutes, after which time 4,4'-dimethoxytrityl chloride (0.8g,1.1 equivalents ) was added.The mixture was again protected from moisture, with a silica gel drying tube and stirred at room temperature for 2.5 hours, monitored by tlc (system B , Rf product 0.45). When the reaction had gone to completion a 1/1 mixture of pyridine/water (9ml) was added and stirring was continued for 30 minutes . More water was then added and the mixture stirred for 2 hours during which time the product precipitated out.

The aqueous phase was extracted with chloroform(5x30ml) and the solution added to the crude product . The combined organic phase was then dried over sodium sulphate and evaporated to dryness. Crystallisation from benzene/pyridine gave *2-N-( 4,4'-Dimethoxytrityl)-2'-deoxyguanosine* (**26**) (1.1g, 100%), m.p.180-182°C, [ Found: C, 65.5; H, 5.3; N, 12.3; O, 16.9%; M (mass spectrum), 568.219591. $C_{31}H_{30}N_5O_6$ calc. C, 65.5; H, 5.3; N, 12.3; O, 16.9%; M, 568.21959]; $\lambda_{max}$ 209nm ( $\varepsilon$ 24,381 $dm^3 mol^{-1} cm^{-1}$), 276 (13,706); $\delta_H$ (200MHz, DMSO-$D_6$ ) 1.7-2.0 (2H, d/m, $C_2$'), 3.3 (18H, broad, water+$C_5$'), 3.75 (6H, s, OMe), 3.7 (1H, m, $C_4$'), 4.1 (1H, m, $C_3$'), 5.5 (1H, t, $C_1$'), 6.8 (3H, m, DMTr), 7.1-7.35 (10H, m, DMTr), 7.6 (1H, s, CH/G), 7.75 (1H, s, G-2-N), 10.6 (1H, s, NH imide); m/z 1137 (polymer? 100%), 722, 639, 568 [M-($H^+$), 70%)

*4-N-( 4,4''-Dimethoxytrityl)-2'-deoxycytidine* (**27**).- 2'-Deoxycytidine (0,5g,2.2mmol) was suspended in dry pyridine ( 8ml) and trimethylsilyl chloride (2.7ml,5equivalents) was added. After stirring 30 minutes 4,4'dimethoxytrityl-chloride ( 1.1equivalents, 0.71g) was added and the reaction mixture was stirred at room temperature overnight, monitored by tlc(system B) Rf product 10/17. When the reaction was complete a 1/1 mixture of pyridine/water (9ml) was added

and stirring continued for 30 minutes . More water(150ml) was then added and the mixture stirred for 2 hours. The aqueous phase was extracted with chloroform(5x30ml) and this solution was dried over sodium sulphate and evaporated to dryness. Crystallisation from benzene gave *4-N-( 4,4"-Dimethoxytrityl)-2'-deoxycytidine* (**27**) (0.35g, 30%), m.p.180-184°C, [Found: C, 68.2; H, 5.7; N, 7.95; O, 18.2%; M (mass spectrum), 528.21343. $C_{30}H_{30}N_3O_6$ requires: C, 68.2; H, 5.7; N, 7.95; O, 18.2%; M, 528.21343]; m/z 600 (70%), 528 [M-($H^+$), 100) , 412, 199, 168, 122, 46, 42

*6-N-(4,4"-Dimethoxytrityl)-2'-deoxyadenosine* (**28**).- 2-'Deoxyadenosine( 0.5g,2mmol ) was suspended in dry pyridine ( 8ml) and trimethylsilyl chloride (2.7ml, 5equivalents) was added. This mixture was stirred at room temperature for 30 minutes monitored by tlc (5% methanol in dichloromethane). When the reacion was complete 4,4'dimethoxytrityl-chloride ( 0.8g, 1.1equivalents) was added and the reaction mixture stirred overnight. Water/pyridine (1/1,10ml) was then added and the solution stirred 40 minutes, followed by addition of more water (120ml) and further stirring 2 hours.The product was extracted into chloroform (5x30ml), dried over sodium sulphate and evaporated to dryness, yielding the solid foam *6-N-(4,4"-Dimethoxytrityl)-2'-deoxyadenosine* (**28**) (0.8g,73%) [ Found: C, 67.4; H, 5.5; N, 12.7; O, 14.5; M (mass spectrum), 552.22470. $C_{31}H_{31}N_5O_5$ requires: C, 67.4; H, 5.5; N, 12.7; O, 14.5%; M, 552.22468]; $\lambda_{max}$ 206nm (Ɛ 31,240$dm^3$ $mol^{-1}$ $cm^{-1}$), 278 (7,293); m/z 706, (silylated, 30%), 552[ M-($H^+$), 100% );

*5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4"-dimethoxytrityl)-2'-deoxyadenosine* (**29**).-
To 6-N-(4,4"-Dimethoxytrityl)-2'-deoxyadenosine (0.602g,1.1mmol), dried by coevaporation from dry pyridine,and dissolved in dry pyridine( 8ml) was added FMOC-Cl ( 1.2 equivalents). The reaction mixture was stirred for 3 hours,monitored by tlc(system A), and when no starting material remained the reaction was poured out into water (50ml) and the products extracted into chloroform(5x30ml). The organic phase was dried over sodium sulphate and evaporated to dryness. Flash chromatography(5% methanol in dichloromethane) yielded the solid foam *5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4"-dimethoxytrityl)-2'-deoxyadenosine* (**29**) (216mg, 25%); $d_H$(200MHz, $CDCl_3$ ), 2.5 (1H, m, $C_2$'), 2.7-2.8 (2H, m, $C_2$'+OH), 3.75 (6H, s, OMe), 4.25 (2H, m,

$C_5$'), 4.4 (3H, m, $C_4$'+$CH_2$), 4.7 (2H, m, $C_3$' +9-fluor), 6.4 (1H, t, $C_1$'), 6.8 (4H, m, DMTr), 7.2-7.5 (14H, m, Fmoc+DMTr), 7.55-7.8 (8H, m, Fmoc), 8.0 (2H, d, adenosine 2 and 9); 8.6 (1H, broad, NH); m/z 775 (mol ion, 80%)

*5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrityl)-2'-deoxyguanosine* (**30**).-

Dry 2-N-(4,4''-dimethoxytrityl)-2'-deoxyguanosine ( 1g,1.8mmol) was dissolved in anhydrous pyridine( 10 ml) and FMOC-Cl ( 1.2equivalents) was added. The reaction mixture was protected from moisture with a silica gel drying tube and stirred at room temperature for 3 hours monitored by tlc(system A) Rf product=0.65. When all starting material had been consumed the reaction was poured out into water and the solid precipitate collected by filtration.

Purification was by flash chromatography ( 20ml methanol in 230ml dichloromethane ) to give the solid foam *5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrityl)-2'-deoxyguanosine* (**30**) (0.7g, 50%) [Found: C, 69.7; H, 5.3; N, 88.35; O, 16.15%; M (mass spectrum), 792.30331. $C_{46}H_{42}N_5O_8$ Requires C, 69.7; H, 5.3; N, 88.35; O, 16.5%.]; $\delta_H$ (200MHz, $CDCl_3$ ), 1.05 (<1H, t, impurity ), 1.8 ( 1H, m, $C_2$'), 2.1 (1H, m, $C_2$'), 2.6 (1H, broad singlet, 3'OH), 3.5 (6H, s, methoxy of DMTr), 3.8 (1H, m $C_3$'), 3.9-4.25 (5H, m, $C_5$', $C_4$', $CH_2$ ), 5.1 (1.2H, s, $H_2O$), 5.6 (1H, t, $C_1$'), 6.6 (1H, d, C-H 9 ), 6.9-7.25 (4H, m, aromatic), 7.3-7.9 (18H, m, aromatic FMOC+DMTr); m/z 792 [M+($H^+$), 90%).

*5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine* (**31**).-

4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine ( 2.0g,3.8mmol) was dried by coevaporation from dry pyridine and dissolved in dry pyridine(16ml) . To this solution FMOC -Cl (1.0g, 1.1equivalents) was added and the mixture was protected from moisture with a silica gel drying tube and stirred at room temperature for 3 hours, monitored by tlc (system A,Rf product = 0.6,Rf starting material = 0.25). When the reaction was complete it was poured into water (200ml) and extracted with chloroform(5x60ml). Purification by flash chromatography (5%methanol in dichloromethane) gave the solid foam *5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine*

(**31**) (0.847g, 30%) [Found: C, 71.8; H, 5.6; N, 5.6; O, 17.0%; M (mass spectrum), 752.29716. $C_{45}H_{42}N_3O_8$ requires C, 71.8; H, 5.6; N, 5.6; O, 17.0%; M, 752.29717 ]; m/z 774 [M+($H^+$), 75%).

*5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4''-dimethoxytrityl)-2'-deoxyadenosine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**32**).- 5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4''-dimethoxytrityl)-2'-deoxyadenosine (463mg, 0.6mmol) was dried by coevaporation from dry pyridine and dichloromethane and then dissolved in dry dichloromethane(12ml). To this solution diisopropylethylamine (0.7ml,2 equivalents) and N,N-diisopropylmethoxyphosphonamidic chloride ( 0.23ml,1.2equivalents) were added. This reaction mixture was stirred under nitrogen for 20 minutes until all starting material had been consumed ( tlc system C,Rfstarting material= 0, Rfproduct= 0.4 ) at which point dry methanol ( 0.01ml) and ethyl acetate, presaturated with nitrogen, ( 20ml) were added . This solution was washed with sodium carbonate solution ( 10% , 2x10ml ) and brine (2x10ml) and after drying over sodium sulphate was evaporated under reduced pressure and precipitated by the addition of a dry toluene solution to a large volume of n- hexane at -20°C. The resulting white solid was collected by filtration and stored under nitrogen at 4°C.

*5'-O-(9-Fluorenylmethoxycarbonyl)-6-N-(4,4''-dimethoxytrityl)-2'-deoxyadenosine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**32**).- (350mg, 64%); $^{31}P$ NMR (90MHz, $CDCl_3$), 149.693 (d )

5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrtyl)-2'-deoxyguanine-3'-O-*N,N-diisopropylmethyl phosphoramidite* (**33**).- 5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrityl)-2'-deoxyguanosine (0.7g,0.9mmol) was dried by coevaporation from dichloromethane containing 10% pyridine and dissolved in dry dichloromethane(2ml). To this was added diisopropylethylamine(0.8ml,2equivalents) and N,N-diisopropylmethoxyphosphonamidic chloride (0.3ml,1.2equuivalents) .This solution was stirred under nitrogen for 20 minutes and monitored by tlc ( system C, product Rf = 0.2). When no starting material remained methanol (0.01ml) and ethylacetate, presaturated with nitrogen, (20ml) were added. The resulting solution

was washed with sodium carbonate solution (10%, 2x10ml) and brine (2x10ml), dried over sodium sulphate and evaporated to dryness. The product was then precipitated by the addition of a dichloromethane solution to a large volume of n-hexane at -20°C to give *5'-O-(9-Fluorenylmethoxycarbonyl)-2-N-(4,4''-dimethoxytrtyl)-2'-deoxyguanine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**33**) (0.578g, 66%); $^{31}$P NMR 149.559 (d).

*5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**34**).- 5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine (0.8g,1.1mmol) was dried by coevaporation from 10% pyridine in dichloromethane(x2) and dissolved in dry dichloromethane(3ml) with diisopropylethylamine( 1.3ml,2equivalents) and N,N-diisopropylmethoxyphosphonamidic chloride (0.4ml, 2 equivalents). This mixture was stirred under dry nitrogen for 15 minutes until no starting material remained(tlc system C, Rf product = 0.33) and dry methanol (0.025ml) and ethyl acetate (40ml), presaturated with dry nitrogen gas, were added. The organic phase was washed with sodium carbonate solution (10%,2x25ml), and brine (2x25ml), dried over sodium sulphate and evaporated to dryness under reduced pressure. The product was precipitated by the addition of a toluene solution to a large volume of cold n-hexane (-70°C) yielding *5'-O-(9-Fluorenylmethoxycarbonyl)-4-N-(4,4''-dimethoxytrityl)-2'-deoxycytidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**34**) (0.584g, 60%); $^{31}$ P NMR 149 (d)

*4-N-Benzoylcytidine* (**35**).- Cytidine (4.2g,17.3mmol) was suspended in dry pyridine (50ml) and chlorotrimethylsilane(30ml,10 equivalents) was added.This mixture was protected from moisture with a silica gel drying tube and stirred at room temperature for 40 minutes, (monitored by tlc system C).

When the reaction was complete benzoyl chloride(12ml ) was added and the reaction was stirred overnight. Pyridine/water 1/1(25ml) was added and stirring continued 40 minutes in an ice bath, monitored by tlc (tlc system B, Rf product = 0.51). The mixture was then evaporated to

dryness under reduced pressure and the crystallisation from water gave *4-N-Benzoylcytidine* (**35**) (3.5g, 60%) [ Found: C, 55.2; H, 5.2; N, 12.1; O, 27.6%; M (mass spectrum), 348.11956. $C_{16}H_{18}N_3O_6$calc. C, 55.2; H, 5.2; N, 12.1; O, 27.6% M, 348.11955 ]; $\delta_H$(200MHz, DMSO-$D_6$), 3.5-3.8 (2H, m, $C_2$'+$C_4$'), 3.8-4.1 (3H, m, $C_5$'+$C_3$'), 5.1-5.2 (3H, broad, 5'+3'+4'OHs), 5.8 (1H, d, $C_1$'), 7.3 (1H, d, C/Ar), 7.4-7.7 (3H, m, Bz), 8.0 (2H, m, Bz), 8.5 (1H, d, C/Ar), 9.8 (1H, broad, NH); m/z 348 [M+($H^+$), 100%), 216 (80).

*3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**36**).- 4-N-Benzoylcytidine (2.556g,7.3mmol) was suspended in dry pyridine(25ml) with dry DMF(6ml) and to this was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane(2.7ml,1.2equivalents). The mixture was protected from moisture with a silica gel drying tube and stirred at room temperature for 1 hour.When no starting material remained( tlc system A, Rf SM =0.2,Rf product = 0.7), triethylammonium bicarbonate solution (2M,15ml) was added and the solution evaporated to dryness.The crude product was redissolved in dichloromethane(150ml) and this solution washed with sodium bicarbonate solution(10ml) and water (30ml),dried over sodium sulphate and evaporated to dryness.The product was coevaporated with dry toluene twice to give the stable stable foam *3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**36**) (3.825g, 86%)

*2'-O-(4-methoxytetrahydropyran-4-yl)-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**37**).- 4-N-Benzoyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (3.5g,6mmol) was dissolved in dry dioxane (30ml) and *p*--toluene sulphonic acid ( 1g,1equivalent) was added.This mixture was stirred at room temperature for 2 hours and 5,6-dihydro-4- methoxy-2H-pyran ( 2.6ml ,4 equivalents) was then added. The reaction was stirred at room temperature a further hour and followed by tlc.(system C, product gave intense purple colour on heating with anisaldehyde.) When the reaction was complete the mixture was neutralised by addition of pyridine (to pH 7) and dichloromethane(200ml) was added.This solution was washed with water(2x40ml) ,dried over sodium -sulphate and evaporated to dryness to givethe oil *2'-O-(4-methoxytetrahydropyran-4-yl)-*

*3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**37**) (3.4682g, 85%) [Found: C, 58.0; H, 7.7; N, 6.0; O, 20.4; Si, 8.0%; M (mass spectrum), 704.33986. $C_{34}H_{54}N_3O_9Si_2$ requires C, 58.0; H, 7.7; N, 6.0; O, 20.4; Si, 8.0%; M, 704.33983]; $\delta_H$ (200MHz, $CDCl_3$), 0.9-1.3 ( 28H, m, TIPDS), 1.75-2.(4H, m, mthp), 3.3 (3H, s, OCH3), 3.6-3.9 (4H, m, mthp), 4.0 (1H, d, $C_4'$), 4.2-4.5 (4H, m, $C_5'+C_3'+C_2'$), 5.8 (1H, s, $C_1'$), 7.4-8.0 ( 6H, m, Bz+cytidinyl H), 8.5 (1H, d, cytidinyl H).

*2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine* (**38**).- 2'-O-(4-methoxytetrahydropyran-4-yl)-3'5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine(3.0g,4.3mmol) was dissolved in dry THF (30ml) and tetrabutylammoniumfluoride (3g in 10ml THF) was added. The mixture was stirred for 10 minutes and the reaction monitored by tlc (system A). When no starting material remained and predominantly a single low running product spot had appeared the solution was evaporated under reduced pressure to dryness,redissolved in dichloromethane(150ml) and washed with water(2x25ml).The organic phase was then dried over sodium sulphate and evaporated to dryness again to give the oil *2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine* (**38**) (1.2g, 61%).

*5'-O-(9-Fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine* (**39**).-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine (1.153g,2.5mmol), dried by coe-vaporation with pyridine was dissolved in anhydrous pyridine (14ml) and stirred in ice. FMOC-Cl (0.711g,1.1equivalents) was added over a period of 20 minutes and the mixture was stirred, protected from moisture with a silica gel drying tube, overnight. Tlc(system A, Rf starting material=0.35,Rfproduct=0.7) revealed reaction had not gone to completion so more FMOC-Cl (0.2g) was added and reaction stirred until no starting material remained.

When the reaction was complete it was poured into water (150ml) and the products extracted with chloroform(5x40ml). The organic phase was dried over sodium sulphate, evaporated to dryness and precipitated by addition of a dichloromethane solution to a large volume of n-hexane. The product was purified by flash chromatography(2-5% methanol in dichloromethane) to give the solid foam *5'-O-(9-Fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine* (**39**) (590mg, 36%) [Found: C, 64.95; H, 6.7; N, 5.3; O, 17.55%; M (mass spectrum), 684.25568. $C_{37}H_{38}N_3O_{10}$ requires C, 64.95; H, 6.7; N, 5.3; O, 17.55%; M, 684.25570]; $_{max}$209nm ( 36,001 $dm^3 mol^{-1} cm^{-1}$), 264nm (24,225): $\delta_H$ (200MHz, $CDCl_3$) 1.7-1.9 (4H, m, mthp), 3.2 (3H, s, OMe), 3.5-3.8 (4H, m, mthp), 4.15 (1H, m, $C_3$'), 4.3 (2H, m, $C_5$'), 4.5 (5H, m, Fmoc$CH_2$+$C_4$'+$C_2$'+Fmoc H9), 6.2 (1H, d, $C_1$'), 7.1-8.0 (15H, m, Bz+Fmoc+Ar/C), 8.75 (1H, broad, NH/C).

*5'-O-(9-fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**40**).- 5'-O-(9-flourenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine(0.5g, 0.7mmol) was dissolved in dry dichloromethane(2ml) with dry diisopropylethylamine(0.7ml, 2equivalents) and the reaction flask was then flushed with nitrogen (approximately 5 minutes). N,N-Diisopropylmethoxyphosphonamidic chloride(0.3ml) was then added and the reaction was stirred for 1 hour, under nitrogen, at the end of which time tlc showed that the reaction was complete (system C, Rf starting material = 0,Rf product = 0.33). Methanol (0.1ml) and nitrogen saturated ethyl acetate (35ml) were then added and this solution was washed first with sodium carbonate solution (10%,2x10ml) and then with brine (2x10ml). The organic phase, dried over sodium sulphate, was evaporated to dryness, under reduced pressure at room temperature, and precipitated by addition of a toluene solution to a large volume of cold hexane(-70°C). Product was purified by flash chromatography(system C) to yield the solid foam *5'-O-(9-fluorenylmethoxycarbonyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-4-N-benzoylcytidine-3'-O-N,N-diisopropylmethyl phosphoramidite* (**40**) (200mg, 35%).

*Methoxyethoxymethyl-triethylammonium chloride* (**41**).- Methoxyethoxymethyl chloride (1ml) was dissolved in dry diethylether(20ml) and dry triethylamine (1.4ml) was added. The reaction was stirred for 16 hours at room temperature after which the product was collected by filtration and dried in vacuo to give *Methoxyethoxymethyl-triethylammonium chloride* (**41**). (1.81g, 100%). The compound is unstable in air (moisture ) so analysis was not attempted.

*Methoxyethoxymethyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**42**).- 3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine (0.68g,1.15mmol) was dissolved in dry acetonitrile,with the MEM salt(0.32g) and this mixture was heated at reflux for 16 hours.The reaction was monitored by tlc (EtOAc/MeOH/NH3 5/1/1 ) Rf starting material = 0.58 product = 0.76. When no more starting material remained the reaction was quenched in water(150ml) and the crude products extracted into chloroform (100ml).The organic phase was dried,over sodium sulphate and evaporated to dryness under reduced pressure. Crystallisation from hexane yielded *4-N-(2-Methoxyethoxymethyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine* (**42**) (0.65g, 20%), m.p. 133-5°C; $\lambda_{max}$ 203nm ( $\varepsilon$ 21,809 $dm^3\ mol^{-1}\ cm^{-1}$), 243nm (10,753), 312nm (14,260); $\delta_H$ 1.0 (28H,m,120,(tipds), 3.25-3.4 (3H, d, O-CH3); 3.6-4.4 (11H, m, $C_2'$, $C_5'$, $C_4'$, $C_3'$, $CH_2$-$CH_2$, OH); 5.75 (2H, m, O-$CH_2$-O); 6.3 (1H, d, $C_1'$), 7.3-7.6(5H, m, Bz), 8.0-8.2 (2H, d of d, cytidyl C-H); m/z 1052, 700 ,678 [M+($H^+$), 85%), 304, 105

Stability of methoxyethoxymethyl protected product .-

1/. Addition of a solution of 3% trichloroacetic acid in dichloromethane to Methoxyethoxymethyl-3'5'-(tetraisopropyldisiloxane-1,3-diyl)-4-benzoylcytidine gave no degradation overnight (detctable by tlc,EtOAc/MeOH/NH3 5/1/1)

2/.Concentrated ammonia gave a mixture of products many of them not uv positive but sugar positive

*4-N-Phthaloylcytidine* (**43**).-Cytidine (1g, 4.1mmol) was suspended in dry pyridine and protected from moisture with a silica gel drying tube and chlorotrimethylsilane(10equivalents) was added

. After stirring for 40 minutes phthaloyl chloride(2equivalents) was added and the reaction mixture was stirred at room temperature for 2 hours. The reaction was quenched in ice and extracted with ethyl acetate (60ml), product tlc (system B, Rf product = 0.26). The organic phase was washed with brine (2x 30ml) and the brine was extracted with a further 2 portions of ethyl acetate (40ml). The organic phases were combined, dried over sodium sulphate, and evaporated to dryness under reduced pressure. Crude product was precipitated by addition of a dichloromethane solution to a large volume of n-hexane yielding *4-N-Phthaloylcytidine* (**43**) (0.2g, 10%).

m/z 590, 574, 518, 446, 356, 314, 259, 242, 103.

The product was unstable to fluoride ions ( tlc system B).

*6-N-Phthaloyladenosine* (**44**).- Adenosine (1g,3.7mmol) was suspended in dry pyridine and chlorotrimethylsilane(10 equivalents) was added. After stirring for 40 minutes phthaloyl chloride (2 equivalents) was added and the reaction mixture, protected from moisture with a silica gel drying tube, was stirred at room temperature for 2 hours. The reaction was then quenched in ice and extracted with ethyl acetate (60ml) which was then washed with brine (2x 30ml). The brine was extracted with a further 2 portions of ethyl acetate (40ml) and the organic phases were combined, dried over sodium sulphate and evaporated to dryness. Crude product (tlc Rf = 0.35 system A) was precipitated by addition of a dichloromethane solution (8ml) to dry hexane(250ml). The precipitate was collected by filtration to give *6-N-Phthaloyladenosine* (**44**) (1.4g, 98%) [Found: C, 54.3; H, 4.05; N, 17.6; O, 24.1%; M (mass spectrum) 398.11007. $C_{18}H_{16}N_5O_6$ requires C, 54.3; H, 4.05; N, 17.6; O, 24.1%; M 398.11005.]; m/z 470, 416, 398 [ M+($H^+$),100%), 358, 350, 338, 308, 292, 284;

*3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-6-N-phthaloyladenosine* (**45**).- 6-N-Phthaloyl adenosine (0.5g,1.2mmol) was dissolved in dry pyridine (15ml) and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.5ml,1.2equivalents) was added. This reaction was protected from moisture with

a silica gel drying tube and stirred for 2.5 hours at room temperature. (tlc system A Rf product =0.83). When the reaction was complete, triethylammonium bicarbonate solution (2M, 5ml) was added and the mixture evaporated to dryness. The residue was redissolved in dichloromethane (100ml) and this solution was washed with sodium bicarbonate solution (saturated 20ml) and water (20ml). After drying, over sodium sulphate, the organic phase was evaporated to dryness and coevaporated with toluene (x2) to obtain a foam of *3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-6-N-phthaloyladenosine* (**45**) (80%) [ Found: C, 56.2; H,6.55; N, 10.9; O, 17.5; Si, 9.05%; M, (mass spectrum), 640.26226. $C_{30}H_{42}N_5O_7Si_2$ Requires: C, 56.2; H, 6.55; N, 10.9; O, 17.5; Si, 9.05%. ]; m/z 731 (M+glycerol, 30%), 639 [M-($H^+$), 70%), 509, 460.

*2'-O-Methoxyethoxymethyl-6-N-phthaloyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-adenosine* (**46**).-6-N-Phthaloyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (3g,4.6mmol) was refluxed in dry ether (75ml) for 20 hours with methoxyethoxymethyl-triethylammonium chloride(4equivalents ). The mixture was then evaporated to dryness and the residue redissolved in chloroform(150ml). This solution was washed with water(2x50ml),dried over sodium sulphate and evaporated to dryness under reduced pressure to give the oil *2'-O-Methoxyethoxymethyl-6-N-phthaloyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-adenosine* (**46**) (2.96g, 87%); $d_H$ (200MHz, $CDCl_3$ ), 1.0 (28H, m, TIPDS), 3.0 (1H, broad, OH), 3.25-3.6 (1H, m, $C_2$'), 4.0-4.6 (4H, m, $C_5$', $C_4$', $C_3$'), 5.2 (<1H, m, water), 6.1 (1H, d, $C_1$'), 7.75-8.1 (4H, d/m, Phth), 8.3 (1H, s, Adenyl), 9.0 (1H, s, Adenyl); m/z 761, 675 [M+($H^+$), 68%), 657, 639, 527, 509, 266, 136.

*Deprotection of 2'-O-Methoxyethoxymethyl-6-N-phthaloyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-adenosine with tetrabutylammonium fluoride* .- 2'-O-Methoxyethoxymethyl-6-N-phthaloyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-adenosine was not stable to tetrabutylammonium fluoride in dichloromethane giving a baseline spot by tlc (system A).

6-N-Phthaloyladenosine was also found to be unstable to tetrabutylammonium fluoride treatment under similar conditions. The product from the reaction was adenosine by tlc (methanol/

ethylacetate/ammonia 5/1/1) Rf=0.22 for both adenosine and the product from treatment of phthaloyl adenosine with tetrabutylammonium fluoride in dry dichloromethane.

*Di-n-butylformamide dimethyl acetal* (**47**).- Di-*n*-butylamine (21ml) and N,N-dimethylformamide dimethyl acetal (18.2ml) were mixed and heated at 100°C for 3 days ,with protection from moisture by a silica gel drying tube. Fractional distillation (bp 158°C at 20mmHg) gave the colourless oil *Di-n-butylformamide dimethyl acetal* (**47**) (40%); $\delta_H$ (200MHz, $CDCl_3$), 0.75-1.0 (6H, m, methyl), 1.1-1.7 (8H, m, methylene), 2.4-2.75 (4H, m, methylene), 3.25 (6H, s, methoxy), 3.4 (1H, s, methine).

*tlc scale reaction of* (**47**)*with adenosine.*- adenosine (50mg) was dissolved in dry dichloromethane (1ml) and di-n-butylformamidedimethylacetal(2 equivalents) was added.The solution was stoppered and set aside for 20 minutes monitored by tlc(system A, Rf starting material = 0.2,Rf product = 0.32). The reaction was complete after 20 minutes. The product was not isolated. This product seemed to be stable to tetrabutylammonium fluoride in dichloromethane for at least 2 days by tlc(system A).

*3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine* (**48**).-Dry adenosine (3g,11.2mmol) was suspended in DMF/pyridine and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (4ml) added dropwise. After stirring at room temperature 1 hour, monitored by tlc(system A, Rf product = 0.44 Rf starting material=0.2, the mixture was neutralised with triethylammonium bicarbonate (2M,10ml) and evaporated to dryness. The residue was taken up in dry dichloromethane(200ml) and this solution washed with saturated sodium bicarbonate solution(40ml) and water (50ml). The organic phase was dried over sodium sulphate and concentrated to a colourless oil. Cystallisation from acetonitrile yielded *3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine* (**48**) (3.04g, 52%), mp 90 °C, [Found: C, 51.8; H, 7.9; N, 13.7; O, 15.7; Si, 11.0%; M (mass spectrum), 510.25677. $C_{22}H_{40}N_5O_5Si_2$ calc. C, 51.8; H, 7.9; N, 13.7; O, 15.7; Si, 11.0%; M, 510.25678]; $d_H$ (200MHz,

$CDCl_3$), 1.1 (28H, m, tipds), 2.1 (1H,s, OH), 2.9 (1H, d, $C_2$'), 3.5 (1H, s, OH), 4.0-2 (2H, m, $C_5$'), 4.6 (1H, d, $C_3$'), 5.1 (1H, m, $C_4$'), 5.8 (2H, s, Adenyl amino), 6.0 (1H, d, $C_1$'), 7.25 (1H, s, $CHCl_3$), 8.0 (1H, s, adenyl), 8.3 (1H, s, adenyl); m/z 510 [M+($H^+$), 90%].

*6-N-Di-n-butylformamidine-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine* (**49**).-3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)adenosine (1g, 2.0mmol) was dissolved in dry dichloromethane (20ml) and di-n-butylformamide dimethylacetal (4 equivalents ) was added. The reaction was protected from light with aluminium foil and moisture by suba-seal and stirred at room temperature for 2 hours under nitrogen ( tlc system A, Rf SM=0.44 Rf product = 0.65 and 0.7). When no more starting material remained more dichloromethane(110ml) was added and the solution washed with saturated sodium bicarbonate (20ml x2 ) .The organic phase was dried over sodium sulphate and evaporated to dryness under reduced pressure. One isomer, the lower by tlc, crystallised from hexane to give *6-N-Di-n-butylformamidine-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine* (**49**) (0.5g, 40%) (Found: C, 57.3; H, 8.8; N, 12.95%. $C_{31}H_{57}N_6O_5Si_2$ requires, C, 57.4; H, 8.7; N, 13.1%.); $\lambda_{max}$ (MeOH), 201nm ( $\varepsilon$ 20,3478 $dm^3mol^{-1}cm^{-1}$), 231nm (13,0637), 314nm (37,5894); $\delta_H$(200MHz, $CDCl_3$), 0.9-1.8 (55H, 3m, TIPDS+DBF), 3.25 (1H, s, 2' OH), 3.3-3.4 (2H, t, $C_5$'), 3.7-3.8 (2H, m, $C_3$'+$C_4$'), 4.0-4.2 (4H, m, $CH_2$ of DBF), 4.6 (1H, d, $C_2$'), 5.2 (1H, t, water), 6.0 (1H, d, $C_1$'), 7.25 (<1H, s, $CHCl_3$), 8.0 (1H, s, CH of DBF), 8.5 (1H, s, Adenyl), 9.0 (1H, s, Adenyl); m/z 755, 663, 649 [M+($H^+$), 50%), 275.

This product can be deprotected by $F^-$ ions(tetrabutylammonium fluoride) in dichloromethane. tlc result(system A), Rf 6-Dinbutylformamidine-3',5'-(tetraisopropyldisiloxane-1,3-diyl)adenosine = 0.65, Rf after fluoride treatment = 0.3, (adenosine = 0.2 same system ).

*5'-O-(4,4"dimethoxytrityl)-2'-deoxycytidine* (**50**).-To a solution of dry 2'-deoxycytidine (0.887g) in anhydrous pyridine was added dimethoxytrityl chloride (1.72g) and the mixture was stirred at room temperature protected from moisture and light for 2 hours. The product mixture was poured into water and extracted into diethyl ether(5x30ml), and evaporated to dryness.The products were separated and purified by wet flash chromatography (5%MeOH in dichloromethane) to give *5'-*

*O-(4,4"dimethoxytrityl)-2'-deoxycytidine* (**50**) (0.783g, 50%); m/z 636, 528 [M-($H^+$), 60%), 110, 107.

*5'-O-(4,4"-Dimethoxytrityl)-2'-deoxycytidine; Reaction with 2,4,-Ditrofluorobenzene( Sangers reagent) in_dichloromethane.-* A sample of 5'-O-(4,4"-dimethoxytrityl)-2'-deoxycytidine was dissolved in dichloromethane and excess dinitrofluorobenzene added. The reaction was monitored by tlc (system A).

A very faint product spot was observed Rf = 0.54, the starting material Rf = 0.15.

Reaction time was 6 hours

*Reaction of Sangers reagent with ethylenediamine.-* Ethylenediamine ( 1ml,17mmol) was dissolved in dry ether and Sangers reagent(3g,17mmol) was slowly added (10 minutes). A violent exothermic reaction took place , and a bright yellow powder was produced which was collected by filtration to give (**51**) (2g, 52%) [Found: C, 42.3; H, 4.8; N, 24.7; O, 28.2%; M, (mass spectrum), 227.07802. $C_8H_{11}N_4O_4$ Requires: C, 42.3; H, 4.8; N, 24.7; O, 28.2%; 227.07802]; m/z 319 (40%), 227[M+($H^+$), 70%].

This product was found to be very insoluble in; $CHCl_3$, water, water / $Na_2CO_3$ 1/1, MeOH very slightly soluble, MeCN, 2M NaOH

tlc (systemA) showed a single low running spot Rf = 0.07

*2'-Deoxycytidine refluxed with ethylenediamine* (**52**).- 2'-Deoxycytidine (0.58g) was dissolved in ethylenediamine(10ml) and this solution was refluxed for 4 hours with protection from moisture with a silica gel drying tube. Reaction monitored by tlc (nBuOH:AcOH:$H_2O$ 3:1:1), Rf SM = 0.44, Rf product = 0.25. The reaction mixture was poured into water (100ml) and washed with diethyl ether(2x20ml).The aqueous phase was evaporated under reduced pressure to give a brown viscous oil (**52**)

*Reaction of* (**52**) *with dinitrofluorobenzene* (**53**).-The oil (**52**) was dissolved in ethanol and to this

solution was added 2,4-dinitrobenzene; a bright yellow precipitate resulted. Attempted liberation of this salt by sodium hydroxide solution (2M) wash of a suspension of (**53**) in chloroform was unsuccessful, the insoluble precipitate remained. m/z 455, 433(100%), 321.

*Reaction of* (**52**) *with 9-fluorenylmethoxycarbonyl- succinimide* (**54**).- (**52**) (0.5g) was dissolved in dry DMF anf FMOC-succinimide (0.6g) added . This mixture was stirred at room temperature overnight and the resulting solid collected by fitration giving (**54**) (0.427g); m/z 493 [M+($H^+$), 60%].

*N-(9-fluorenylmethoxycarbonyl)-6-aminohexan-1-ol* (**55**).- 6-Aminohexan-1-ol (0.35g,2,96mmol) was dissolved in dry pyridine (10ml) and 9-fluorenylmethoxycarbonyl-succinimide (1g, 2.96mmol) was added. This mixture was stirred at room temperature overnight,protected from moisture with a silica gel drying tube (tlcsystem A, Rf product = 0.55). The reaction was poured into water and extracted with chloroform (4x40ml), dried over sodium sulphate and evaporated to dryness to give solid *N-(9-fluorenylmethoxycarbonyl)-6-aminohexan-1-ol* (**55**) (0.588g, 58%) [Found: C, 74.1; H, 7.7; N, 4.1; O, 14.1%; M (mass spectrum), 340.19128. $C_{21}H_{26}O_3N$ requires C, 74.1; H, 7.7; N, 4.1; O, 14.1%; M, 340.19126]; $\lambda_{max}$ 209nm ( $\varepsilon$ 35,466$dm^3 mol^{-1} cm^{-1}$), 264nm (17,068); $\delta_H$ (200 MHz, $CDCl_3$), 1.1-1.5 (5H, m, methylene), 2.9 (2H, d, $CH_2$-N), 3.4 (2H, t, $CH_2$-O), 5.2 (2H, broad, water), 6.25 (2H, s, $CH_2$Fmoc), 6.7 (1H, s(broad), N-H), 7.3 (4H, m, Fmoc), 7.8-7.9 (4H, m, Fmoc); m/z 340, 246, 179,

*N-(9-Fluorenylmethoxycarbonyl)-6-aminohexan-1-ol-1-O-N,N-diisopropylcyanoethylphosphoramidite* (**56**).-N-(9-Fluorenylmethoxycarbonyl)-6-aminohexan-1-ol (100mg,0.3mmol) was dissolved in dry dichloromethane and dry diisopropylamine (0.1ml,2equivalents) was added. The reaction vessel was flushed with dry oxygen free nitrogen and chlorocyanoethyl-N,N-diisopropylphosphoramidite (0.08ml,1.2equivalents) was added. The reaction mixture was stirred under nitrogen for 1.5 hours until the reaction appeared to be complete by tlc (system C, Rf product =0.78. One drop of dry methanol was added followed by

ethyl acetate (10ml). This solution was washed with sodium carbonate solution (10%, 2ml) and brine (2ml) ,dried over sodium sulphate and evaporaed to dryness to give the oil *N-(9-Fluorenylmethoxycarbonyl)-6-aminohexan-1-ol-1-O-N,N-diisopropylcyanoethylphosphoramidite* (**56**) (130mg, 80%).

The product from this rection was reacted with deblocked sarcosyl resin and then the resin was treated with 10% DBU in dry dichloromethane .The resulting resin was then found to give a positive result when tested for primary amines(Kaiser test). This resin turned bright yellow upon treatment with dinitrofluorobenzene.

*N-(2,4-dinitrophenyl)-6-aminohexan-1-ol* (**57**).- 6- Aminohexan-1-ol (0.8g,6.8mmol ) was dissolved in ether and 2,4-dinitrofluorobenzene(1.3g,1equivalent) was added. The resulting yellow solid, tlc Rf product = 0.52 (tlc system A) was collected by filtration giving *N-(2,4-dinitrophenyl)-6-aminohexan-1-ol* (**57**) (1.3g, 70%) [ Found: C, 50.7; H, 6.4; N, 14.8; O, 28.15%; M (mass spectrum), 284.12462. $C_{12}H_{18}N_3O_5$ requires C, 50.7; H, 6.4; N, 14.8; O, 28.15%; M, 284.12463]; $_H$ (200MHz, $CDCl_3$), 1.1-2.0 (9H, m, $CH_2$ +OH), 3.25-.75 (4H, m, $CH_2$ each end), 6.9 (1H, d, sangers 6), 8.25 (1H, d of d, sangers 5), 8.5 (1H, broads, N-H), 9.1 (1H, d, sangers 3); m/z 284 [M+($H^+$), 80%), 217.

*1-O-(4,4'-dimethoxytrityl)-6-N-(2,4-dinitrophenyl)-6-aminohexan-1-ol* (**58**).- N-(2,4-dinitrophenyl)-6-aminohexan-1-ol( 1g) was dissolved in dry pyridine (15ml ) and DMTr -Cl (1equivalent ) added. The reaction was protected from moisture with a silica gel drying tube and stirred at room temperature for 1 hour and monitored by tlc(system A, Rf starting material = 0.48,Rf product = 0.78). When the reaction was complete water (150ml) was added and the products extracted into chloroform(5x30ml). The organic phase was dried over sodium sulphate and evaporated to dryness at reduced pressure. Purification was by flash chromatography(250ml dichloromethane /5ml triethylamine) to give the oil *1-O-(4,4'-dimethoxytrityl)-6-N-(2,4-dinitrophenyl)-6-aminohexan-1-ol* (**58**) (60%) [Found: C, 67.7; H, 6.0; N, 7.2; O, 19.1%; M (mass spectrum), 585.24744 $C_{33}H_{55}N_3O_7$ requires C, 67.7; H, 6.0; N, 7.2; O, 19.1%; M, 585.24748]; $I_{max}$ 3400$cm^{-1}$, 1600, 1400;

$_H$ (200MHz, $CDCl_3$), 1-3.5 (12H, m, alkyl $CH_2$), 3.75 (6H, s, methoxy ), 6.8 (5H, m, DMTr), 7.2-5 (9H, m, DMTr +Sangers 6 ), 8.25, (1H, d, Sangers 5), 8.5 (1H, broad, N-H ), 9.1 (1H, d, Sangers 3); m/z 585 [M-($H^+$),100%].

*Attempted N-benzoylation of* (**58**).- (**58**) (0.6g ) dissolved in dry pyridine (15ml ) and benzoyl chloride (2 equivalents, 0.25ml ) was added dropwise. The reaction was protected from moisture with a silica gel drying tube and stirred at room temperature for 1 hour, monitored by tlc (dichloromethane/triethylamine 5/1)

No reaction was observed .Product was starting material (**57**); $\nu_{max}$ 3400$cm^{-1}$ (OH).

Also no reaction after addition of triethylamine.

*N-(2,4-dinitrophenyl)-6-aminohexan-1-ol-1-O-N,N-diisopropylcyanoethylphosphoramidite* (**59**).- 6-N-(2,4-dinitrophenyl)-6-aminohexan-1-ol (140mg ) was dissolved in dry THF(5ml) with dry diisopropylethylamine (0.1ml ) and 2-cyanoethyl-N,N- diisopropylchlorophosphoramidic chloride ( 0.132 ml 1.2 equivalents ). When the reaction was complete by tlc (system C,Rf product 0.8,Rf sm = 0.39) methanol (5 drops ) and ethyl acetate (20ml ,nitrogen saturated ) were added . The organic phase was washed with sodium carbonate (10%,5ml x2 ) and brine(5ml x 2 ), dried over sodium sulphate and evaporated to dryness under reduced pressure. The product was purified by flash column chromatography (EtOAc/dichloromethane,1/1) to give the oil *N-(2,4-dinitrophenyl)-6-aminohexan-1-ol-1-O-N,N-diisopropylcyanoethylphosphoramidite* (**58**) (100mg, 80%); $^{31}P$ NMR 147.21( s ).

*3-N-Dinitrophenyl-3-amino-1,2-propanediol.* (**60**).- 3-Amino-1,2-propanediol (0.5g, 5.5mmol) was dissolved in methanol (50ml) and to this was added 2,4-dinitrofluorobenzene (1.0g, 5.5mmol). This solution was set aside overnight. The solution was then evaporated under reduced pressure and triturated with diethyl ether (the insoluble orange oil being retained). Further evaporation evaporation yielded an orange oil *3-N-Dinitrophenyl-3-amino-1,2-propanediol* (**59**) (1g, 78%); m/z 258[ M+($H^+$), 80%).

*1-O-(4,4'-Dimethoxytrityl)-3-(dinitrophenyl)-3-amino-1,2-propanediol* (**60**).- 3-N-Dinitrophenyl-3-amino-1,2-propanediol (0.5g,1.94mmol) was dissolved in anhydrous pyridine (12ml) and to this solution was added 4,4'-dimethoxytrityl chloride (0.722g, 2.1mmol). The mixture was protected from moisture with a calcium chloride guard tube and stirred at room temperature for 2 hours. The mixture was then poured into water (100ml) and the product extracted with chloroform (4x 30ml). After drying over sodium sulphate, the solution was evaporated to dryness. Purification was by flash chromatography (3% methanol in dichloromethane ) to give the yellow oil *1-O-(4,4'-Dimethoxytrityl)-3-(dinitrophenyl)-3-amino-1,2-propanediol* (**61**) (0.7g, 64% ); $\delta_H$ (200MHz, $CDCl_3$) 2.4 (1H, s, OH), 3.25-3.6 (4H, m, $C_1$,$C_3$), 3.75 (6H, s, OMe), 4.1 (1H, m, $C_2$), 5.3 (1H, s, water), 6.8-7.5 (14H, m, trityl+DNP 5), 8.2 (1H, m, DNP 6), 8.75 (1H, m, NH), 9.1 (1H, m, DNP 3 ); m/z 558 [M+($H^+$), 100%].

| Oligonucleotide | Synthesis cycle | 5' Protection | 5' Deblocking reagent | End procedure |
|---|---|---|---|---|
| (**a**) $T_4$ | Phos 1 (Table 4) | DMTr | 3% TCA/ dichloromethane | dep-phos1 (Table 5a) |
| (**b**) $T_4$ | Phos 2 (Table 5) | FMOC | 10% DBN/ dichloromethane | dep-phos1 |
| (**c**) test 1 | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (**d**) 489A | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (**e**) d $(A)_8$ | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (**f**) d $(A)_8$ | ssme103a | FMOC | 10% DBU/ acetonitrile | depr03 |
| (**g**) d $(A)_8$ | ssce103a | FMOC | 10% DBU/ acetonitrile | depr03 |
| (**h**) $T_6$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (**i**) $T_6$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (**j**) $T_6$ | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (**k**) $T_6$ | PIPCE1 | FMOC | 20% piperidine/ acetonitrile | deprce03 |

| Oligonucleotide | Synthesis cycle | 5' Protection | 5' Deblocking reagent | End procedure |
|---|---|---|---|---|
| (l) $T_6$ | ssce103a | FMOC | 10% DBU/ acetonitrile | deprce03 |
| (m) d $(A)_8$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (n) d $(A)_{20}$ | ssce103a | DMTr | 3% TCA dichloromethane | deprce03 |
| (o) d $(GT)_3$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (p) d $(GT)_3$ | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (q) d $(GA)_3$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (r) d $(GA)_3$ | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| (s) d $(C)_6$ | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (t) | PIPME1 | FMOC | 20% piperidine/ acetonitrile | depr03 |
| (u) | ssce103a | DMTr | 2% TCA dichloromethane | deprce03 |
| (v) r $(C)_6$ | RNA2P | FMOC | 20% piperidine/ acetonitrile | depr03 |

| Oligonucleotide | Synthesis cycle | 5' Protection | 5' Deblocking reagent | End procedure |
|---|---|---|---|---|
| **(w)** | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| **(x)** | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |
| **(y)** | ssce103a | DMTr | 3% TCA/ dichloromethane | deprce03 |

# References

1. T.Cech, *Science,* **231**, 470
2. Oligonucleotide Synthesis : a practical approach. M.J.Gait, (editor), IRL press, 1984.
3. Merri ield, *J. Am. Chem. Soc.*, 1963, **85**, 2149-2154.
4. R.L.Letsinger and V.Mahadevan, *J. Am. Chem. Soc..*, 1965, **87**, 3526-7.
5. M.J.Gait, M.Singh, R.C.Sheppard, M.D.Edge, A.R.Greene, G.R.Heathcliffe, T.C.Atkinson, C.R.Newton, A.F.Markham, *Nucleic Acids Res..*, 1980, **8**, 1081.
6. T.Atkinson and M.Smith, in Oligonucleotide Synthesis : a practical approach., IRL Press, 1984.
7. J.Katzhendler, S.Cohen, E.Rahamim, M.Weisz, I.Ringel, J.Deutsch, *Tetrahedron*, 1989, **9**, 2777-2792.
8. R.T.Pon, N.Usman, K.K.Ogilvie, *Biotechniques*, 1988, **6**, 768.
9. H.G.Khorana, G.M.Tenor, J.G.Moffatt, E.H.Pol, *Chem. Ind. (London)*, 1956, 1523.
10. H.G.Khorana, W.E.Razzell, P.T.Gilham, G.M.Tenor, E.H.Pol, *J. Am. Chem. Soc.*, 1957, **79**, 1002.
11. C.B.Reese, *Tetrahedron*, 1978, **34**, 3143-3179.
12. R.L.Letsinger, V.Mahadevan, *J. Am. Chem. Soc.*, 1966, **88**, 5319.
13. F.Eckstein, I.Rizk, *Angew. Chem., Int. Edn. Engl.*, 1967, **6**, 695.
14. A.F.Cook, *J. Org. Chem.,* 1968, **33**, 3589.
15. C.B.Reese, R.Arentzen, *J. Chem. Soc. Perkin Trans. 1,* 1977, 445.
16. C.B.Reese, R.C.Titmas, L.Yau,*Tetrahedron Lett.,* 1978, **30**, 2727-2730.
17. G.Silber, D.Flockerzi, R.S.Varma, R.Charubala, E.Uhlmann, W.Pfleiderer, *Helv. Chem. Acta,* 1981, **64**, 1704.
18. F.Himmelsbach, B.S.Shulz, T.Trichtinger, R.Charubala, W.Pfleiderer, *Tetrahedron,* 1981, **40**, 59.
19. C.B.Reese, A.Ubasawa, *Tetrahedron Lett.,* 1980, **21**, 2265-2268.
20. R.L.Letsinger, J.L.Finnan, G.A.Heavner, W.B.Lunsford, *J .Am. Chem. Soc.,* 1975, **97**, 3278.
21. S.L.Beaucage, M.H.Caruthers,*Tetrahedron Lett.,* 1981, **22**, 1859.

22. S.P.Adams, K.S.Kavka, E.J.Wykes, S.B.Holder, G.R.Gallupi, *J. Am. Chem. Soc.,* 1983, **105**, 661.

23. L.J.McBride, M.H.Caruthers, *Tetrahedron Lett.,* 1983, **24**, 245.

24. X.Gao, B.L.Gaffney, M.Senior, R.R.Riddle, R.A.Jones, *Nucleic Acids Res.,* 1985, **13**(2), 573.

25. N.D.Sinha, J.Biernat, J.McManus, H.Koster, *Nucleic Acids Res.,* 1984, **12**, 4539.

26. C.Claesen, G.I.Tesser, C.E.Dreef, J.E.Marugg, G.A.van der Marel, J.H.van Boom, *Tetrahedron Lett.,* 1984, **25**, 1307.

27. M.H.Caruthers, *Reactive Polymers, Ion exchangers, Sorbents,* 1987, **6**, 159.

28. R.M.Hall, A.Todd, R.F.Webb, *J. Chem. Soc.,* 1957, 3291.

29. M.Sekine, H.Mori, T.Hata, *Tetrahedron Lett.,* 1979, 1145

30. P.J.Garegg, T.Reyberg, J.Stawinsky, R.Stromberg, *Chimica Scripta,* 1985, **25**, 280.

31. B.C.Froehler, M.D.Matteuci, *Tetrahedron Lett.,* 1985, **27**, 469.

32. B.C.Froehler, P.G.Ng, M.D.Matteuci, *Nucleic Acids Res.,* 1986, **14**(13), 5399.

33. J.E.Marugg, M.Tromp, E.Kuyl-Yeheskiely, G.A.van der Marel, J.H.vanBoom, *Tetrahedron Lett.,* 1986, **27**(23), 2661.

34. E.Kuyl-Yeheskiely, M.Spierenburg, H.van der Elst, M.Tromp, G.A. van der Marel, J.H.van Boom, *Recl. Trav. Chim. Pays-Bas,* 1986, 505-506

35. A.B.I. user bulletin/communication.

36. G.A.Allante, *Proc. Amer. Assoc. Canc. Res.*, May 1987.

37. G.M.Blackburn, J.S.Cohen, A.J.Todd, *J.Chem.Soc.(C),* 1966, 239-245.

38. A.Zwierak, *Synthesis*, 1975, 507-509.

39. B.C.Froehler, *Tetrahedron Lett.,* 1986, **27**(46), 5575-5578.

40. F.Benseler, L.W.McLaughlin, *Synthesis,* 1986, 45.

41. R.Lohrman, D.Soll, H.Hayatsu, E.Ohtsuka, H.G.Khorana. *J.Am.Chem.Soc.*, 1966, **88**, 819.

42. G.S.Ti, B.L.Gaffney, R.A.Jones, *J. Am. Chem.Soc.,* 1982, **104**,1316-9.

43. T.Hata, T.Kurihara, *Chem. Lett.,* 1973, 859.

44. H.Takaku, T.Shimida, Y.Morita, T.Hata, *Chem. Lett.*, 1976, 19.

45. A.V.Steinfield, F.Naider, J.M.Becker, *J.Chem.Res.,Miniprint,* 1979, 1437.

46. J.Igolen, C.Morin, *J.Org. Chem.*, 1980, **45**, 4802.

47. J.C.Schulhof, D.Molko, R.Teoule, *Nucleic Acids Res.*, 1987, **15**(2), 397.

48. H.Schaller, H.G.Khorana, *J. Am. Chem. Soc.*, 1963, **85**, 3828.

49. M.D.Matteuci, M.H.Caruthers, *J. Am. Chem. Soc.*, 1981, **103**, 3185.

50. A.Kume, R.Iwase, M.Sekine, T.Hata, *Nucleic Acids Res.*, 1984, **12**(2), 8525.

51. B.C.Froehler, M.D.Matteuci, *Nucleic Acids Res.*, 1983, **11**(22), 8031.

52. L.H.Koole, P.J.L.M.Quaedflieg, W.H.A.Kuijpers, N.L.H.L.Broeders, H.A.Langermans, M.H.P.van der Genderen, H.M.Buck, *Chemistry Proceedings B*, 1988, **91**(2),

53. J.Heikkila, J.B.Chattopadhyaya, *Acta Chem. Scand. B.*, 1983, **37**, 263-265.

54. M.Fujii, S.Yamakage, H.Takaku, T.Hata,*Tetrahedron Lett.*, 1987, **28**(46), 5713-5716.

55. C.A.A.Claesen, A.M.A.Pistorius, G.I.Tesser,*Tetrahedron Lett.*, 1985, **26**(32), 3859-3862.

56. M.Mag, J.Engels, *Nucleic Acids Res.*, 1988, **16**(8), 3525.

57. H.Schaller, G.Weiman, B.Lerch, H.G.Khorana, *J. Am. Chem. Soc.*, 1963, **85**, 3821.

58. J.B.Chattopadhyaya, C.B.Reese, *J. Chem. Soc. Chem. Commun.*, 1978, 639.

59. E.Ohtsuka, M.Ikehara, D.Soll, *Nucleic Acids Res.*, 1982, **10**, 6553.

60. S.A.Narang, *Tetrahedron*, 1983, **39**, 3.

61. M.Sekine, T.Hata, *J. Am. Chem. Soc.*, 1986, **108**, 4581-4586.

62. J.H. van Boom, P.M.J.Burgers, C.H.M.Verdagaal, G.Wille,*Tetrahedron,* 1978, **34**, 1999-2007.

63. J.B.Chattopadhyaya, C.B.Reese, A.H.Todd, *J. Chem. Soc., Chem.Commun.*, 1979, 987.

64. N.Balgobin, S.Josephson, J.B.Chattopadhyaya,*Tetrahedron Lett.*, 1981, **22**(37), 3667-3670.

65. S.Josephson, N.Balgobin, J.B.Chattopadhyaya,*Tetrahedron Lett.*, 1981, **22**, 4537-4540.

66. Y.Ma, E.Sonveaux., *Nucleosides, Nucleotides,* 1987,**6**(1&2), 491-493.

67. N.Balgobin, J.B.Chattopadhyaya, *Nucleosides, Nucleotides,* 1987, **6**(1&2), 461-463.

68. C.Lehmann, Y.Z.Xu, C.Christodoulou, Z.K.Tan, M.J.Gait, *Nucleic Acids Res.*, 1989, **17**(7), 2379.

69. N.Usman, K.K.Ogilvie, M-Y.Jiang, R.J.Cedergren, *J. Am. Chem. Soc.*, 1987, **109**, 7845-7854.

70. W.T.Markiewicz, *J.Chem.Research (S),* 1979, 24-25.

71. W.E.Parham, E.L.Anderson, *J. Am. Chem. Soc.*, 1948, **70**, 4187.

72. R.Kierzek, M.H.Caruthers, C.E.Longfellow, D.Swinton, D.H.Turner, S.M.Frier, *Biochem istry,* 1986,**25**, 7840.

73. T.Kamamura, M.Tsuchiya, K.Koura, M.Sekine, K.Shinozaki, K.Miura, T.Hata, *J. Am. Chem. Soc.*, 1984, **106**, 4552-4557.

74. H.Tanimura, T.Fukazawa, M.Sekine, T.Hata, J.W.Efcavitch, G.Zon, *Tetrahedron Lett.*, 1988, **29**(5), 577-578.

75. B.E.Griffin, M.Jarman, C.B.Reese,*Tetrahedron,* 1968, **24**, 639.

76. C.B.Reese, R.Saffhill, J.E.Sulston, *Tetrahedron,* 1970, **26**,1023-1030.

77. C.B.Reese, P.A.Skone, *Nucleic Acids Res.*, 1985, **13**, 5215.

78. C.B.Reese, H.T.Serafinowska, G.Zappia,*Tetrahedron Lett.*, 1986, **27**(20), 2291-2294.

79. M.M.Kreevoy, R.W.Taft, *J. Am. Chem. Soc.*, 1955, **77**, 5590.

80. T.Wu, K.K.Ogilvie, R.T.Pon, *Nucleic Acids Res.*, 1989, **17**(9), 3501.

81. S-H.Chou, P.Flynn, B.Reid, *Biochemistry*, 1989, **28**, 2422-2435.

82. H.Takaku, K.Kamaike, H.Tsuchiya, *J. Org. Chem.*, 1984, **49**, 51.

83. T.Tanaka, M.Orita, S.Uesugi, M.Ikehara, *Tetrahedron,* 1988, **44**(14), 4331-4338

84. T.Takaku, T.Ito, S.Ueda, *J. Org. Chem.*, 1986, **51**, 931.

85. T.Kempe, W.I.Sundquist, F.Chow, S.Hu, *Nucleic Acids Res.*, 1985, **13**(1), 45.

86. L.M.Smith, S.Fung, M.W.Hunkapiller, T.J.Hunkapiller, L.E.Hood, *Nucleic Acids Res.*, 1985, **13**(7), 2399.

87. A.Murakami, J.Tada, K.Yamagata, J.Takano, *Nucleic Acids Res.*, 1989, **17**(14), 5587.

88. M.S.Urdea, B.D.Warner, J.A.Running, M.Stempien, J.Clyne, T.Horn, *Nucleic Acids Res.*, 1988, **16**(11), 4937.

89. J.L.Fourrey, J.Varence, C.Blonski, P.Dousset, D.Shire, *Tetrahedron Lett.*, 1987, **28**(43), 5157-5160.

90. C.B.Reese, P.A.Skone, *J. Chem. Soc. Perkin Trans.1*, 1984, 1263-1271.

91. W.L.Sung, *J. Org. Chem.*, 1982, **47**, 3623-3628.

92. A.F.Maggio, M.Lucas, J-L,Barasat, J-L.Imbach, *Tetrahedron Lett.*, 1984, **25**, 3195-3198.

93. B.S.Sproat, A.Lamond, B.Beijer, P.Neuner, U.Ryder, *Nucleic Acids Res.*, 1989, **17**(9), 3373.

94. J.Harambidis, M.Chai, G.W.Tregear, *Nucleic Acids Res.*, 1987, **15**(12), 4857.

95. C.Vincent, P.Tchen, M.Cohen-Solal, P.Kourilsky, *Nucleic Acids Res.*, 1982, **10**(21), 6787.

96. D.E,Draper, L.Gold, *Biochemistry,* 1980, **19**,1774-1781.

97. L.M.Smith, J.Z.Sanders, R.J.Kaiser, P.Hughes, S.Dod, C.R.Connell, C.Heiner, S.B.H.Kent, L.E.Hood. *Nature (London),* **321**, 674.

98.

a. T.Kempe, W.I.Sundquist, F.Chow, *Nucleic Acids Res.*, 1985, **13**, 45.

b. T.Tanaka, S.Tamatsukuri, M.Ikehara, *Tetrahedron Lett.*, 1987, **28**, 2611.

c. J.M.Coull, H.L.Weith, R.Bischoff, *Tetrahedron Lett.*, 1986, **27**, 3991.

d. S.Agarwal, C.Christodolou, M.J.Gait, *Nucleic Acids Res.*, 1986, **14**, 6227.

99. B.A.Connelly, *Nucleic Acids Res.*, 1987, **15**, 3131.

100. V.K.Kansel, T.Huynh-Dinh, J.Igolen, *Tetrahedron Lett.*, 1988, **29**(43), 5537-5540.

101. P.S.Nelson, R.Sherman-Gold, R.Leon, *Nucleic Acids Res.*, 1989, **17**(18), 7179.

102. P.S.Nelson, R.A.Frye, E.Liu, *Nucleic Acids Res.*, 1989, **17**(18), 7187.

103. A.M.Alves, D.Holland, M.D.Edge, *Tetrahedron Lett.*, 1989, **30**(23), 3089-3092.

104. L.A.Carpino, G.Y.Han. *J.Am.Chem.Soc.*, 1970, **92**, 5748-5753.

105. E.Atherton, C.Burg, R.C.Sheppard, B.J.Williams, *Tetrahedron Lett.*, 1979, **20**, 3041.

106. C.Gioeli, J.Chattopadhyaya, *J. Chem. Soc., Chem. Commun.*, 1982, 672

107. J.E.Baldwin, J.Cutting, W.Dupont, L.Kruse, L.Silberman, R.C.Thomas, *J.C.S.Chem.Commun.*, 1976, 736.

108. X.Gao, B.L.Gaffney, M.Senior, R.R.Riddle, R.A.Jones. *Nucleic Acids Res.*, 1985, **13**(2), 573.

109. G.Turner, PhD University of Edinburgh 1989.

110. R.Arentzen, Y.T.Yan Kui, C.B.Reese, *J.Chem.Soc.,Chem.Commun.*, 1975, 509.

111. G.R.Owen, C.B.Reese, *J.Chem.Soc.(C)*, 1970, 2401.

112. E.J.Corey, J-L.Gras, P.Ulrich, *Tetrahedron Lett.*, 1976, **11**, 809-812.

113. E.Kaiser, *Anal. Biochem.*, 1970, **34**, 595 -598.

114. L.A.Carpino, G.Y.Han, *J. Org. Chem.*, 1972, **37**, 3405

# Appendix A

## Synthesis Cycles

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: SSCE103A
NUMBER OF STEPS: 89
DATE: Sep 21, 1989
TIME: 16:40

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | #18 To Waste | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 9 | #18 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 28 | Phos Prep | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 8 | 19 | B+TET To Col 1 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | 19 | B+TET To Col 1 | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | 9 | #18 To Column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | 20 | B+TET To Col 2 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 20 | B+TET To Col 2 | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 9 | #18 To Column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | 21 | B+TET To Col 3 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 90 | TEI to column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 28 | 21 | B+TET To Col 3 | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 29 | 9 | #18 To Column | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 30 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 31 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 32 | 16 | Cap Prep | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 33 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 34 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 35 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 36 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 37 | 22 | Cap To Col 1 | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 38 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 39 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 40 | 23 | Cap To Col 2 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 41 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 43 | 24 | Cap To Col 3 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

(Continued next page.)

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: SSCE103A
NUMBER OF STEPS: 89

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 45 | 4 | Wait | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 46 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 47 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 48 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 49 | 81 | #15 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 50 | 13 | #15 To Column | 19 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 51 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 52 | 10 | #18 To Waste | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 53 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 54 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 55 | 9 | #18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 56 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 57 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 58 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 59 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 60 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 61 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 62 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 63 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **64** | **33** | **Cycle Entry** | **1** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 65 | 10 | #18 To Waste | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 66 | 9 | #18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 67 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 68 | 1 | Block Flush | 6 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 69 | 6 | Waste-Port | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 70 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 71 | 82 | #14 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 72 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 73 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 74 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 75 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 76 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 77 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 78 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 79 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 80 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 81 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 82 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 83 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 84 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yess | Yes | No |
| 85 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 86 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 87 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 88 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 89 | 7 | Waste-Bottle | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: ssme103
NUMBER OF STEPS: 86
DATE: Sep 21, 1989
TIME: 16:52

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | #18 To Waste | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 9 | #18 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 28 | Phos Prep | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Ye s |
| 8 | 19 | B+TET To Col 1 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | 20 | B+TET To Col 2 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | 21 | B+TET To Col 3 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | 4 | Wait | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | 16 | Cap Prep | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 28 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 29 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 30 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 31 | 22 | Cap To Col 1 | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 32 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 33 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 34 | 23 | Cap To Col 2 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 35 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 36 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 37 | 24 | Cap To Col 3 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 38 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 39 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 40 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 41 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 43 | 81 | #15 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

(Continued next page.)

SYNTHESIS CYCLE
VERSION 1.34



CYCLE NAME: ssme103
NUMBER OF STEPS: 86

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | 13 | #15 To Column | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 45 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 46 | 10 | #18 To Waste | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 47 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 48 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 49 | 15 | #13 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 50 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 51 | 15 | #13 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 52 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 53 | 15 | #13 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 54 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 55 | 15 | #13 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 56 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 57 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **58** | **33** | **Cycle Entry** | **1** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 59 | 18 | #16 To Waste | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 60 | 12 | #16 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 61 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 62 | 1 | Block Flush | 6 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 63 | 6 | Waste-Port | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 64 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 65 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 66 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 67 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 68 | 82 | #14 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 69 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 70 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 71 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 72 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 73 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 74 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 75 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 76 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 77 | 14 | #14 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 78 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 79 | 18 | #16 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 80 | 12 | #16 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 81 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 82 | 18 | #16 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 83 | 12 | #16 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 84 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 85 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 86 | 7 | Waste-Bottle | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: PIPME1
NUMBER OF STEPS: 85
DATE: Sep 21, 1989
TIME: 16:45

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | #18 To Waste | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 9 | #18 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 28 | Phos Prep | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 8 | 19 | B+TET To Col 1 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | 20 | B+TET To Col 2 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | 61 | TET To Waste | 2 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | 21 | B+TET To Col 3 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 9 | #18 To Column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | 4 | Wait | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | 16 | Cap Prep | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 28 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 29 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 30 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 31 | 22 | Cap To Col 1 | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 32 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 33 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 34 | 23 | Cap To Col 2 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 35 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 36 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 37 | 24 | Cap To Col 3 | 8 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 38 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 39 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 40 | 10 | #18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 41 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 43 | 81 | #15 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

(Continued next page.)

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: PIPME1
NUMBER OF STEPS: 85

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | 13 | #15 To Column | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 45 | 4 | Wait | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 46 | 10 | #18 To Waste | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 47 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 48 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 49 | 9 | #18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 50 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 51 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 52 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 53 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 54 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 55 | 9 | #18 To Column | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 56 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 57 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **58** | **33** | **Cycle Entry** | **1** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 59 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 60 | 9 | #18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 61 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 62 | 1 | Block Flush | 6 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 63 | 6 | Waste-Port | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 64 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 65 | 82 | #14 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 66 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 67 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 68 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 69 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 70 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 71 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 72 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 73 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 74 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 75 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 76 | 14 | #14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 77 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 78 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 79 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 80 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 81 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 82 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 83 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 84 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 85 | 7 | Waste-Bottle | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: RNA2P
NUMBER OF STEPS: 93
DATE: Oct 17, 1988
TIME: 13:59

| STEP NUMBER | FUNCTION £ | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | £18 To Waste | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 9 | £18 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 28 | Phos Prep | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 90 | TET to column | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 8 | 19 | B+TET To Col 1 | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | 19 | B+TET To Col 1 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | 19 | B+TET To Col 1 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 10 | £18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | 90 | TET to column | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 20 | B+TET To Col 2 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | 20 | B+TET To Col 2 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 20 | B+TET To Col 2 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 10 | £18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 28 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 29 | 90 | TET to column | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 30 | 21 | B+TET To Col 3 | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 31 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 32 | 21 | B+TET To Col 3 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 33 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 34 | 21 | B+TET To Col 3 | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 35 | 90 | TET to column | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 36 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 37 | 4 | Wait | 300 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 38 | 16 | Cap Prep | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 39 | 10 | £18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 40 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 41 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | +45 | Group 1 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 43 | 22 | Cap To Col 1 | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

(Continued next page.)

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: RNA2P
NUMBER OF STEPS: 93

| STEP NUMBER | FUNCTION £ | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | -46 | Group 1 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 45 | +47 | Group 2 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 46 | 23 | Cap To Col 2 | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 47 | -48 | Group 2 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 48 | +49 | Group 3 On | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 49 | 24 | Cap To Col 3 | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 50 | -50 | Group 3 Off | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 51 | 4 | Wait | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 52 | 10 | £18 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 53 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 54 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 55 | 81 | £15 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 56 | 13 | £15 To Column | 23 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 57 | 4 | Wait | 45 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 58 | 10 | £18 To Waste | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 59 | 2 | Reverse Flush | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 60 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 61 | 9 | £18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 62 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 63 | 9 | £18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 64 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 65 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **66** | **33** | **Cycle Entry** | **1** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** |
| 67 | 10 | £18 To Waste | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 68 | 9 | £18 To Column | 20 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 69 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 70 | 1 | Block Flush | 6 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 71 | 6 | Waste-Port | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 72 | 5 | Advance FC | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 73 | 82 | £14 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 74 | 14 | £14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 75 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 76 | 14 | £14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 77 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 78 | 14 | £14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 79 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 80 | 14 | £14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 81 | 2 | Reverse Flush | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 82 | 14 | £14 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 83 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 84 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 85 | 10 | £18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 86 | 9 | £18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| 87 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 88 | 7 | Waste-Bottle | 1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

(Continued next page.)

SYNTHESIS CYCLE
VERSION 1.34

CYCLE NAME: RNA2P
NUMBER OF STEPS: 93

| STEP NUMBER | FUNCTION £ | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 89 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 90 | 10 | £13 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 91 | 9 | £13 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 92 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 93 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

END PROCEDURE
VERSION 1.34



PROCEDURE NAME: deprce03
NUMBER OF STEPS: 27
DATE: Sep 24, 1989
TIME: 10:18

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 8 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | 8 | Flush To CLCT | 9 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 27 | #10 To Collect | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 8 | Flush To CLCT | 9 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | 1 | Block Flush | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 42 | #10 Vent | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

END PROCEDURE
VERSION 1.34

PROCEDURE NAME: depr03
NUMBER OF STEPS: 44
DATE: Sep 24, 1989
TIME: 10:19

| STEP NUMBER | FUNCTION # | NAME | STEP TIME | STEP ACTIVE FOR BASES A | G | C | T | 5 | 6 | 7 | SAFE STEP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2 | 26 | #8 To Column | 40 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 3 | 30 | #17 To Waste | 7 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 4 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 5 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 6 | 1 | Block Flush | 3 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 7 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 8 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 9 | 30 | #17 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 10 | 11 | #17 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 11 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 12 | 11 | #17 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 13 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 14 | 11 | #17 To Column | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 15 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 16 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 17 | 27 | #10 To Collect | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 18 | 2 | Reverse Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 19 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 20 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 21 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 22 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 23 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 24 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 25 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 26 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 27 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 28 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 29 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Ye |
| s | | | | | | | | | | | |
| 30 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 31 | 27 | #10 To Collect | 18 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 32 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 33 | 1 | Block Flush | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 34 | 4 | Wait | 900 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 35 | 8 | Flush To CLCT | 9 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 36 | 27 | #10 To Collect | 15 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 37 | 8 | Flush To CLCT | 9 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 38 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 39 | 1 | Block Flush | 4 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 40 | 10 | #18 To Waste | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 41 | 9 | #18 To Column | 30 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | 2 | Reverse Flush | 60 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 43 | 1 | Block Flush | 10 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 44 | 42 | #10 Vent | 5 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |