SYNTHESIS OF PHOSPHONOACETATE RNA AND A TWO-STEP RNA SYNTHESIS

APPROACH

By

Brian G. Stell

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This thesis entitled:

SYNTHESIS OF PHOSPHONOACETATE RNA AND A TWO-STEP RNA SYNTHESIS

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Written By Brian G. Stell

has been approved for the Department of Biochemistry

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Marvin H. Caruthers

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Robert Kuchta

*Date*_____

The final copy of this thesis has been examined by the signatories, and we

Find that both the content and the form meet acceptable presentation standards

Of scholarly work in the above mentioned discipline.

Stell, Brian G. (Ph.D., Biochemistry, Department of Chemistry and Biochemisty)

Synthesis of Phosphonacetate RNA and A Two-Step RNA Synthesis Approach

Thesis directed by Distinguished Professor Marvin H. Caruthers

Phosphonoacetate RNA dimers and oligomers were synthesized using solid phase synthesis. Two different, orthoganol approaches were used for the 2' and 5' oxygens of the phosphoramidite synthons in order to maintain the phosphonoacetate linkage upon 2'-O deprotection. The first utilized a 2'-O-silyl protection (fluoride labile) along with a 5'-O-DMT protection (acid labile). The second path used an inverse 2'-O-ACE (acid labile) protecting group along with a 5'-O-silyl protection strategy. An additional emphasis in this dissertation was on the solid phase synthesis of unmodified RNA using a two-step cycle. The synthesis was performed using a 5'-O-carbonate-2'-O-1,3-Benzodithiolylium (BDT) protected synthon. During synthesis a peroxy anion buffer removes the 5'-carbonate protection and simultaneously oxidizes the internucleotide phosphite linkage. Following synthesis and removal from support the 2'-O-BDT group was removed. All products were then isolated and characterized.

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CHAPTER I

INTRODUCTION

1.1. Four Step Synthesis of DNA/RNA

The phosphoramidite approach to synthesize DNA on a solid support as developed by Caruthers in the 1980's (Beaucage and Caruthers 1981; Matteucci and Caruthers 1981) is illustrated in **Figure 1.1**.

1) **Deprotection** TCA/CH$_2$Cl$_2$

2) **Condensation** Tetrazole/CH$_3$CN

Protected deoxynucleoside phosphoramidite

3) **Capping** Ac$_2$O/N-Me-Imid THF/Py

4) **Oxidation** Iodine/H$_2$O/THF/Py

Phosphotriester internucleotide bond

Phosphitetriester internucleotide bond

B=A^{Bz}, G^{iBu}, C^{Ac} & U

= Solid support

DMT = 4,4'-dimethoxy-triphenylmethyl
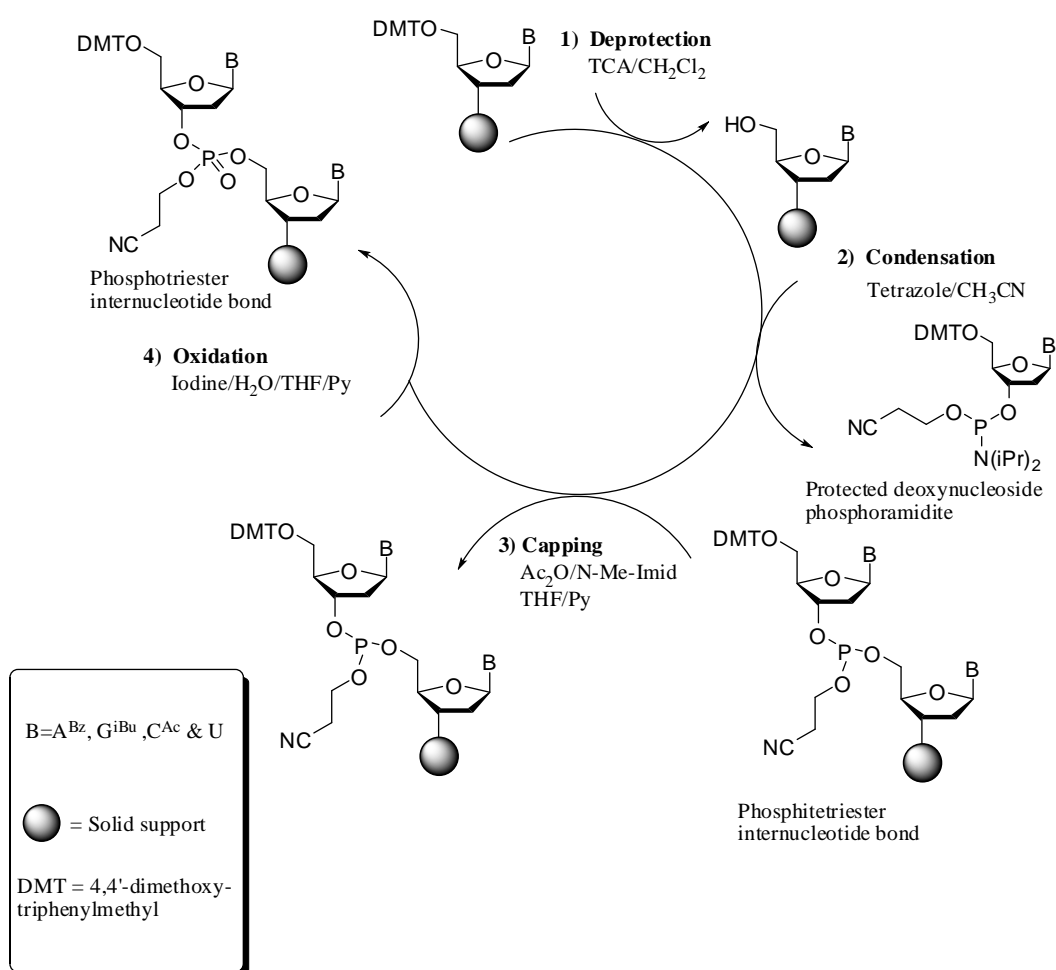
Figure 1.1: **Standard Four-Step Solid Phase Synthesis Cycle for Preparing Oligodeoxyribonucleotides (TCA: Trichloroacetic Acid; Bz: Benzoyl; iBu: Isobutyryl; Ac: Acetyl; Solid Support: Controlled Pore Glass or Polystyrene).**

In general, the strategy is an iterative cycle that builds the oligodeoxyribonucleotide (oligomer) on an insoluble support derivatized with the first 2'-deoxyribonucleoside and elongated one base at a time. The cycle begins with removal of the 5'-protecting group (step 1). This is followed by coupling (step 2) where a 2'-deoxyribonucleoside-3'-phosphoramidite is joined to the 5'-hydroxyl of the support-linked 2'-deoxyribonucleoside to form a phosphite triester. Unreacted 5'-hydroxyls are then masked by acetylation via a procedure commonly referred to as capping (step 3) and finally the phosphite triester is oxidized (step 4) to a phosphate triester. The oligomer is then extended by further repetitions of the cycle. The 2'-deoxyribonucleoside monomers used in solid phase DNA synthesis require four orthogonal chemical reactivities in order to eliminate unwanted side reactions during iterations of the synthesis cycle. These areas of orthogonality are the following: 5'-hydroxyl protection where this blocking group is removed following each round of synthesis; nucleobase protection to prevent various side reactions such as condensation of the incoming synthon with unprotected oxygen or exocyclic amine functionalities; phosphate protection; and an additional level of orthogonality in the form of the linker to the solid support. In some instances several of these groups can be removed following synthesis by using the same reagent, examples of which will be described later in this section. Exhaustive work has been done over the past 20 years, modifying nearly every detail of the above described synthesis cycle in order to optimize purity, efficiency, and scalability and to produce oligodeoxyribonucleotide analogs. In this section, the three most successful analogous strategies will be described using the same basic chemistries to synthesize RNA.

Solid phase RNA synthesis has proven to be a remarkable challenge because additional orthogonality is required due to the presence of the 2'-hydroxyl group. A significant problem

encountered in attempting to synthesize high quality RNA is an effective combination of 5'-and

2'-protecting groups. Because the 5'-protecting group is removed following each round of

synthesis, 2' protection, also on a hydroxy group, must be extremely stable to the reagent chosen

for removal of the 5' group. Furthermore, because the 2'-protecting group is in close proximity

to the reaction center (i.e. the 3' hydroxyl), the steric load on the 2'-hydroxyl is a concern.

Another aspect of extreme importance is the method for removal of the 2'-blocking group. Under

basic and acidic conditions, RNA is subject to strand cleavage in addition to 5'-3' to 5'-2'

migration via a transesterification reaction (**Figure 1.2**) (Li and Breaker 1999). In order to

maintain
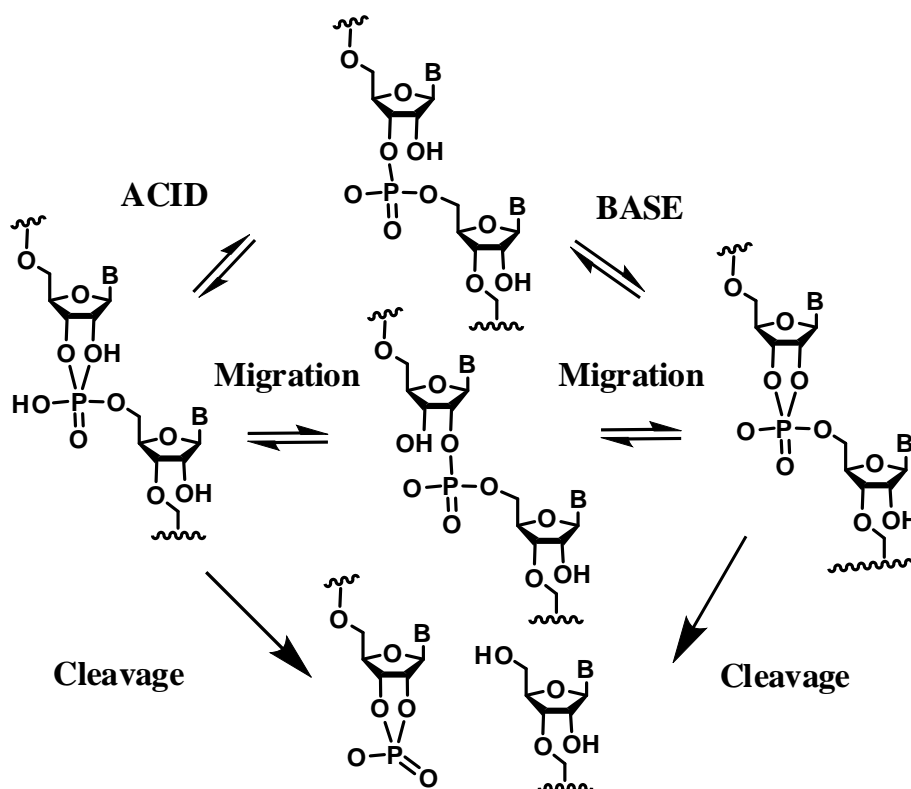
Figure 1.2: **Mechanisms of Acid and Base Catalyzed Cleavage and Migration (Transesterification).**

5'-3' connectivity throughout the RNA oligomer and to prevent strand cleavage, base labile protection as well as protecting groups removed under strongly acidic conditions must be avoided.

One early synthesis scheme developed by Ogilvie and co-workers (Wu, Ogilvie et al. 1989) utilized the traditional 4',4'-dimethoxytriphenylmethyl (DMT) on the 5'-hydroxyl, a *t*-butyldimethylsilyl (TBDMS) on the 2'-hydroxyl, and appropriate base-labile nucleobase protection (**Figure 1.3**).
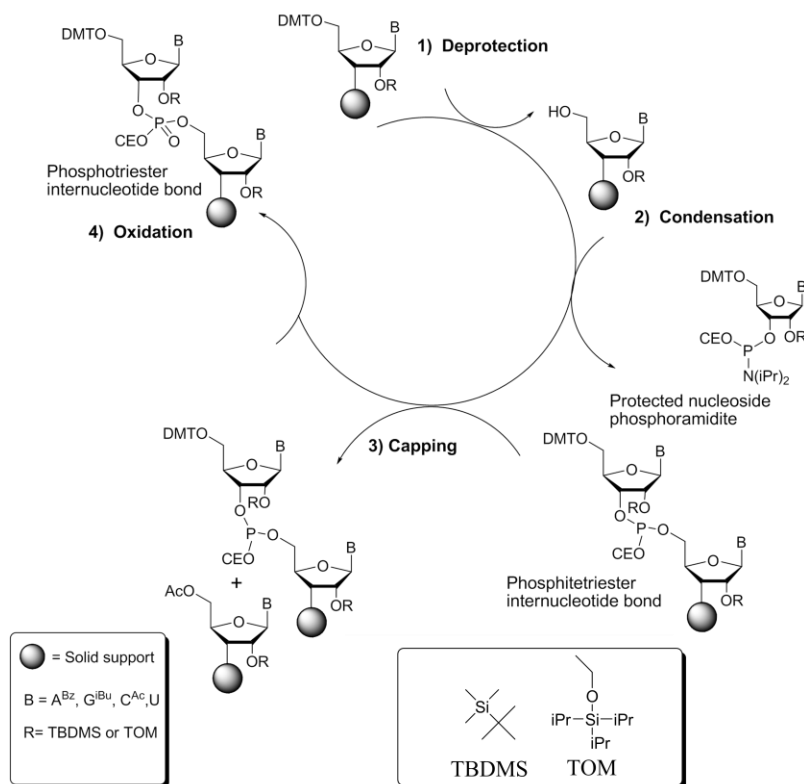
Figure 1.3: **Structure of the Triisopropylsiloxylmethyl (TOM) and, t-Butyldimethylsilyl**

(TBDMS)   **Protecting Groups and the Synthesis Cycle Used to Prepare Silyl Protected RNA.**

DMT is extremely labile to acid while TBDMS is stable to strong acid and base, but is labile to fluoride ion. The solid support was controlled pore glass (CPG) with the 3'-terminal nucleoside attached via a base labile succinyl linker (Matteucci and Caruthers 1981). The phosphate protecting group, β-cyanoethyl, and the nucleobase protecting groups are base labile. Therefore, after chain assembly using this cycle, the oligomer can be cleaved from support with a nucleophilic base that simultaneously removes protecting groups from the nucleobases and phosphate, and generates RNA having only 2' hydroxyl protection. Removal of the TBDMS group can be accomplished using a nucleophillic fluoride ion such as tetrabutylammonium fluoride (TBAF).

Protection with a silyl group on the 2'-hydroxyl is useful because it is stable to both acid and nucleophilic base. This strategy allows for the use of acid following each round of synthesis to deprotect the 5'-hydroxyl. Following synthesis, nucleophillic base can be used to remove phosphate and nucleobase protecting groups and to cleave the oligomer from the support. The 2'-silyl group can then be removed from the oligomer as the final step (Wincott, DiRenzo et al. 1995). Disadvantages with this strategy include low coupling yields and a 2'-protected oligomer that is very lipophillic due to the hydrophobicity of the TBDMS group. Thus the synthetic RNA is only partially water soluble (Marshall and Kaiser 2004) which leads to slow, incomplete protecting group cleavage under aqueous fluoride conditions. A related disadvantage is that preparation of the ribonucleoside 3'-phosphoramidite inevitably generates variable amounts of the 2'-isomer. This isomer also reacts in the synthesis cycle and generates contaminating, unnatural 2'-5' internucleotide linkages.

An improvement to the TBDMS strategy was to use the triisopropylsiloxylmethyl (TOM) group on the 2'-hydroxyl (**Figure 1.3**) (Pitsch S 2001) TOM group is compatible with the DMT group and does not alter the synthesis scheme outlined in **Figure 1.3**. The TOM group is also removed with TBAF after cleavage and deprotection. The main improvement to the TOM group was higher coupling yields which resulted in improved RNA quality with less rigorous purification. The improvemens in yield has been hypothesized to be due to less steric bulk local to the 3'-hydroxyl during phosphite triester bond

formation (Pitsch S 2001). The main disadvantage for the TOM chemistry is the synthesis of the

protected ribonucleoside monomers. The synthesis of these monomers requires considerable technical

expertise and even under these conditions, low yields of final synthons results in the generation of

expensive, protected ribonucleoside phosphoramidites.

An innovative approach to RNA synthesis was published by Caruthers and Scaringe in the late

90's. This method outlined an approach whereby an acid labile 2'-protecting group was used in

conjunction with a fluoride labile group on the 5'-hydroxyl (Scaringe, Wincott et al. 1998). On the 2'-

hydroxyl, *bis*(2-acetoxyethoxy)-methyl orthoester (ACE) was used (**Figure 1.4**). This group
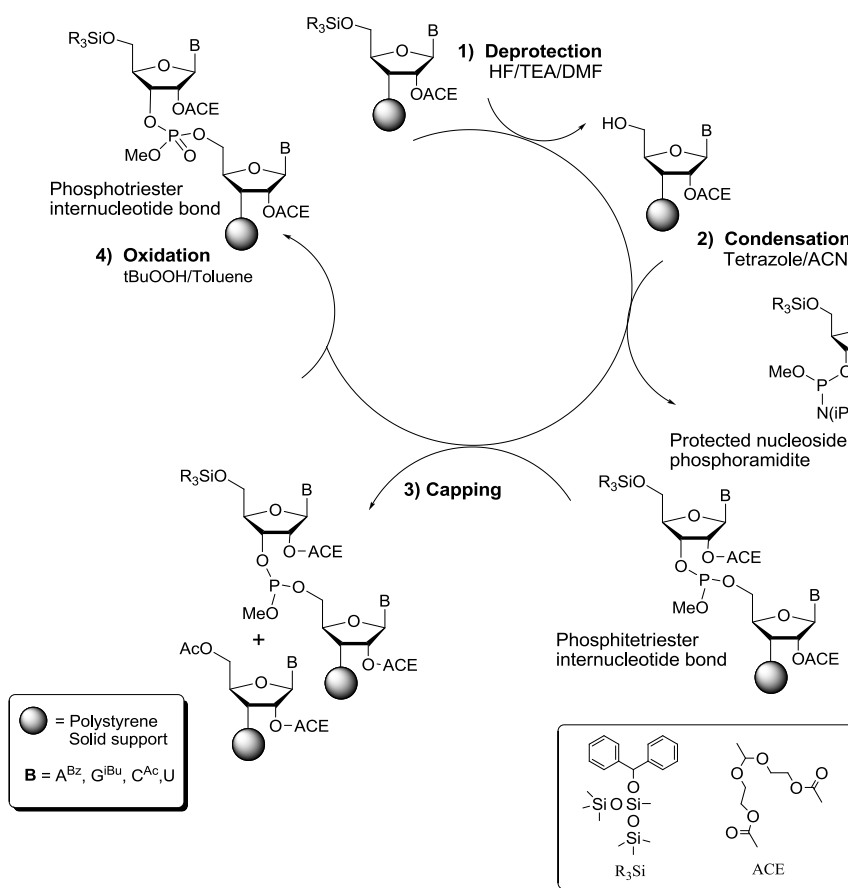
Figure 1.4: **Structure of the *Bis*(2-acetoxyethoxy)-methyl orthoester (ACE) Protecting Group and the Synthesis Cycle Used to Prepare ACE Protected RNA.**

was converted to *bis*(2-hydroxyethoxy)-methyl orthoester upon cleavage from the support with

methylamine which renders the protecting group 10 times more labile to acid. Following this conversion,

only a mild solution of acetic acid in water at pH 3.8 was needed to completely remove the 2'-protecting

group. Because fluoride ion degrades the controlled pore glass (CPG) support, this methodology required

the use of a polystyrene support. Additionally fluoride reacts with the β-cyanoethyl protecting group

commonly used on phosphorus. Therefore a methyl group, which is inert towards fluoride, was used as

protection on phosphate. This substitution was advantageous as higher coupling yields were routinely

observed with methyl phosphoramidites relative to β-cyanoethyl. Removal of the methyl group required

one additional deprotection step using a dithiolate salt (Dahl 1990) and was carried out post-synthesis

with synthetic RNA still bound to the solid support. Also by unmasking the two hydroxyls on the ACE

group, the 2'-protected RNA oligomer was water soluble which enhanced final yields through increased

recovery of the RNA. In summary, the acid-labile ACE group was stable to nucleophilic base and

fluoride ion. This allows for successive rounds of treatment with fluoride ion to remove the 5'-hydroxyl

protection while still using base-labile nucleobase protection on the ribonucleoside monomers. Following

synthesis and cleavage from support the 2'-protecting group can be safely removed with mild acid.

1.2.   Two-Step Synthesis of Oligodeoxyribonucleotides

In collaboration with Agilent Laboratories, the Caruthers Laboratory recently developed a novel

two-step method for synthesizing oligodeoxynucleotides on a solid support (Sierzchala, Dellinger et al.

2003). This methodology (**Figure 1.5**) arose from a need to fulfill two new, specific applications for

synthetic oligodeoxynucleotides. These applications were the use of DNA as a potential therapeutic,

which
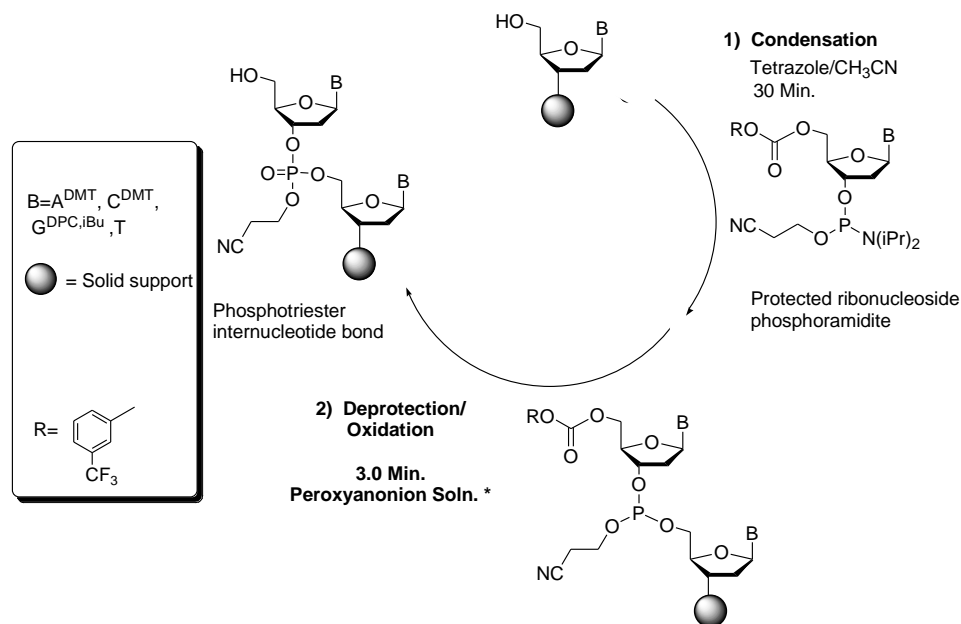
Figure 1.5: **Two-Step Solid Phase Synthesis Cycle Used to Synthesize Oligodeoxynucleotides (DPC; Diphenylcarbamoyl).**

required large scale synthesis, and microarrays having DNA imbedded on a two dimensional glass surface. The standard four step cycle is only marginally useful for these two applications. This is because the DMT cation generated during acid deprotection was a reversible reaction under anhydrous conditions. The reversible nature of this reaction resulted in failure sequences having one or more random deletions. This acid treatment also yielded side-products caused by cleavage of synthetic DNA due to acid depurination of deoxyadenosine and deoxyguanosine. These side-products, which were shorter in length than the product, can represent a significant amount of impurities. Depurination created a larger problem when occurring on planar surfaces because the resulting side-products cannot be removed. Thus, after microarray synthesis was complete, the final product was used while support-bound and, due to depurination, was present in low yield. In order to overcome this problem, a peroxy anion labile, aryl carbonate protecting group was used on the 5'-hydroxyl group. This strategy eliminated the

use of strong acid and hence depurination during deprotection. The reaction by which the aryl carbonate was removed results in carbon dioxide, a phenolate and the free 5'-hydroxyl, an irreversible reaction under standard laboratory conditions. Thus there was no possibility for the same side-products (deoxyoligonucleotides with deletions) as observed during acid detritylation. Additionally, by using a peroxy anion solution to deprotect the 5'-hydroxyl directly after phosphoramidite coupling (**Figure 1.5**), the phosphite triester was rapidly oxidized. As a consequence two steps: oxidation and 5'-deprotection, were combined relative to the standard four step cycle shown in **Figure 1.1**.

The synthesis of 5'-aryl carbonate-2'-deoxynucleoside 3'-phosphoramidites was facile and typically resulted in high yields. Standard nucleobase protecting groups could not be used on adenosine and cytidine as the peroxy anion solution was sufficiently nucleophilic to remove the standard acetyl and benzoyl groups from N^4-2'-deoxycytidine and N^6-2'-deoxyadenosine respectively. Therefore DMT was used to N-protect 2'-deoxyadenosine and 2'-deoxycytidine, an orthogonal protecting group because this synthesis strategy did not utilize the acid labile DMT. Due to the omission of a capping step in the two-step synthesis cycle, it was also necessary to protect O^6 on the nucleobase of deoxyguanosine because, in the absence of a capping step, phosphoramidite addition to O^6 was known to occur (Suzuki, Ohsumi et al. 1994). Therefore, in addition to an isobutyryl group on N^2, N,N-diphenylcarbamoyl was used to protect 2'-deoxyguanosine on the O^6 oxygen. In summary, the two-step cycle used a carbonate protecting group on the 5' hydroxyl which was removed following each round of synthesis with a basic solution of peroxy anions. Peroxy anion-stable protecting groups were therefore used on the nucleobases and removed following synthesis during cleavage of the oligomer from the support.

A major focus of the research outlined in this thesis was adaptation of this two-step synthesis cycle for use in solid phase RNA synthesis. An important requirement was finding a suitable 2'-protecting group compatible with the peroxy anion solution. Also the two-step RNA chemistry was to be

designed for use on a planar glass surface. Therefore a silyl-based protecting group strategy as outlined in

Figure 1.4 was incompatible, because deprotection of the 5'-group required the use of fluoride ion, which

degrades glass materials. Chapter 4 discusses the use of benzodithiol-2-y1 as a protecting group for the 2'

hydroxyl group and its application to the two-step synthesis of RNA.

1.3.   Synthesis and Biochemical Properties of Phosphonoacetate Oligodeoxynucleotides

Recent work in the Caruthers Laboratory on oligodeoxynucleotide alkylphosphonates has resulted

in the synthesis of DNA containing a phosphonoacetate internucleotide linkage (Dellinger, Sheehan et al.

2003). This analog has acetic acid functionality in place of one of the non-bridging oxygens of the

internucleotide linkage (**Figure 1.6**). The resulting phosphorus center is chiral with a pKa of

approximately 3.4, rendering it isoelectric with a natural phosphodiester bond. DNA oligomers
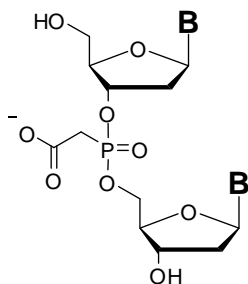
Figure 1.6:   **Structure of the Phosphonoacetate Dinucleotide.**

having the phosphonoacetate modification were synthesized using protected 2'-deoxynucleoside acetic

acid phosphinoamidites. The synthesis of the phosphine is shown in **Figure 1.7**. Bromoacetyl bromide
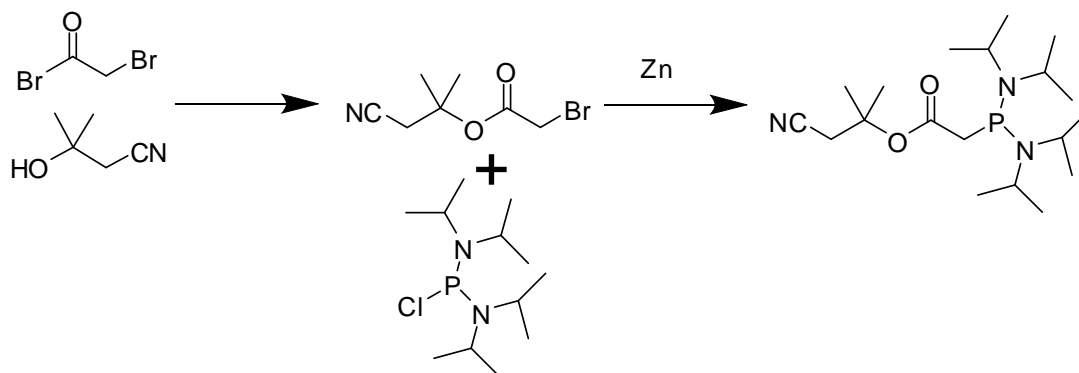
Figure 1.7: **Sythesis of Acetic Acid, [bis(*N,N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl Ester.**

was reacted with a suitable alcohol, in this case 3-hydroxy-3-methylbutyronitrile, to afford the properly

protected acetyl bromide. After purification, 1,1-dimethylcyanoethyl bromoacetate was reacted with

bis(*N,N*-diisopropylamino)chlorophosphine in the presence of granular zinc via a Reformatskii reaction to

form the acetic acid, [*bis*(*N,N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl ester. The 5'-

DMT and *N*-protected 2'-deoxynucleosides of adenine, guanine, cytosine and thymine were reacted with

[*bis*(*N,N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl ester using 1-H-tetrazole to form the

acetic acid phosphinoamidites as shown in **Figure 1.8**.
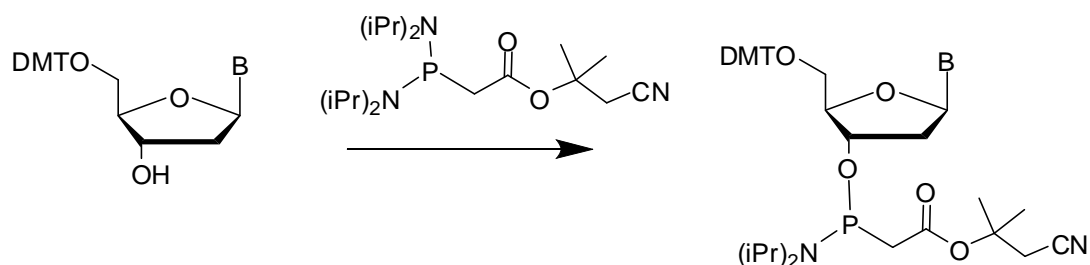
Figure 1.8: **Preparation of Protected Deoxyribonucleoside Phosphinoamidites.**

The solid phase synthesis cycle for preparing phosphonoacetate DNA is shown in **Figure 1.9**. This
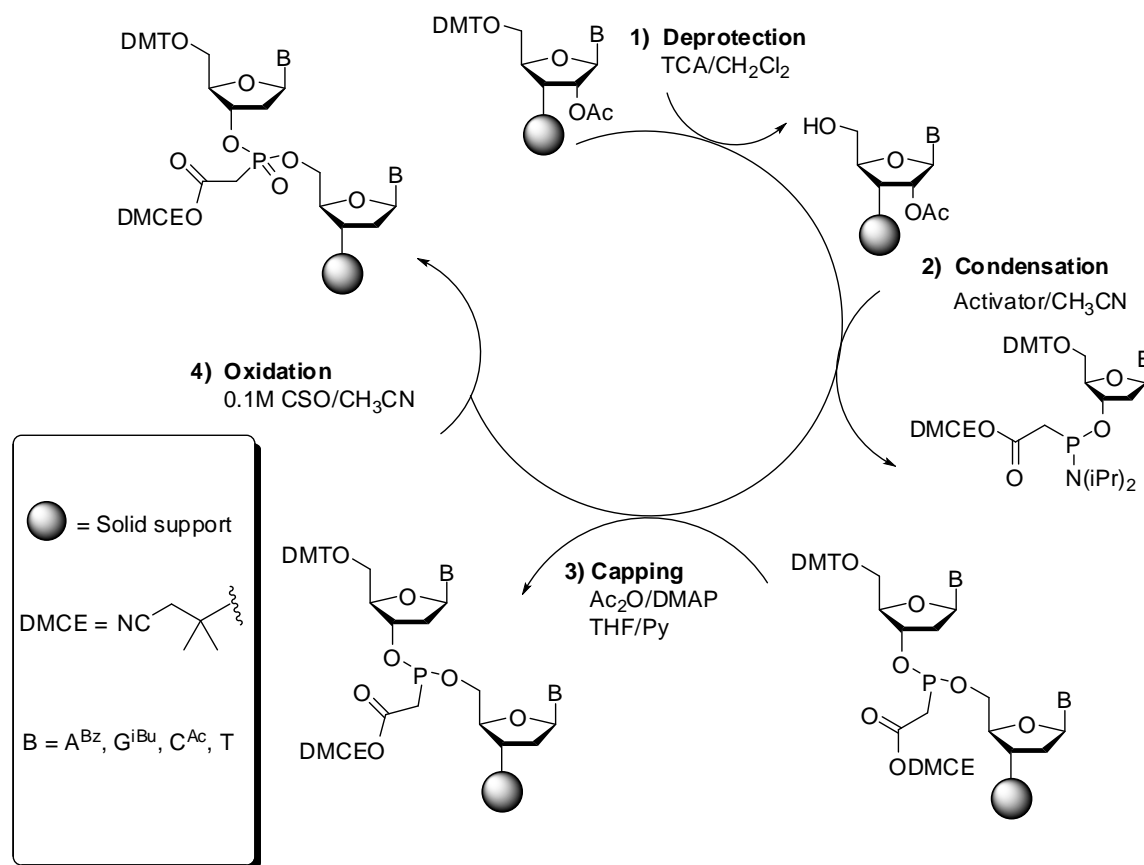
Figure 1.9: **Solid Phase Synthesis Cycle Used to Synthesize Phosphonoacetate**
Oligodeoxyribonucleotides.

cycle was adapted from procedures used to prepare methylphosphonate DNA (Hogrefe, Vaghefi et al.

1993). The key differences when compared to a standard DNA synthesis cycle (**Figure 1.9**) were the

coupling times, oxidation conditions, and capping solution. Best results were achieved when the coupling

time was increased to 1998 seconds (33 minutes or the maximum allowed on an ABI 394 automated

DNA synthesizer) to achieve coupling yields typically above 97% with 1H-tetrazole as the activating

agent. The capping reagent was modified by using 4,4-dimethylaminopyridine and pyridine in

tetrahydrofuran to prevent de-alkylation of the protecting group on phosphorus (Hogrefe, Vaghefi et al.

1993). The oxidation conditions were kept anhydrous by using (1S)-(+)-10-(camphorsulfonyl)oxaziridine

in anhydrous acetonitrile and reacting for 180 seconds. After chain elongation, the ester protecting the

acetic acid moiety was removed via β-elimination with a 1.5% solution of the hindered base 1,8-diazabicyclo(5.4.0)undec-7-ene in acetonitrile while the oligomer was still support-bound. After ester deprotection, the oligomer was treated with 40% aqueous methylamine in order to deprotect the nucleobases and free the oligomer from the support (Reddy, Hanna et al. 1994).

The biochemical properties of phosphonoacetate oligomers have been examined in considerable detail. Fully modified phosphonoacetate DNA forms stable duplexes with complementary, unmodified RNA. Based upon circular dichroism, this heteroduplex adopts the A-form configuration (Sheehan et. al.). Additionally, phosphonoacetate DNA:RNA duplexes activate ribonuclease RNase H which leads to the degradation of the RNA strand of the duplex. When exposed to snake venom phosphodiesterase and DNase I, fully modified phosphonoacetate oligomers are completely stable (Dellinger, Sheehan et al. 2003).

The synthesis of the phosphine used for preparing the 2'-deoxynucleoside phosphinoamidites (**Figure 1.7**) has proven to be quite versatile as the same procedures were used to prepare various acetic acid esters having many additional alkyl groups. For example, methyl, n-butyl and phenyl were used to partially mask the charges on a 2'-deoxythymidine phosphonoacetate homopolymer 12 bases in length. The ability of these partially modified 12mers to enter cells in the absence of any transfecting agents such as cationic lipids was studied. When 12mers having no modifications were exposed to the cells, no transfection was seen without the use of cationic lipids. However when the cells were treated with the esterified phosphonoacetate oligomers bearing only 3 or 5 charges, they reversibly diffused into cells (Yamada 2003).

These results prompted us to attempt the synthesis of phosphonoacetate RNA because of the immense potential for siRNA, miRNA and aptamers as therapeutic reagents and for their use to control gene expression of cells in culture (Kurreck 2003). We envisioned synthesizing phosphonoacetate RNA and utilizing its potential to be esterified as a possible solution to the problem of oligonucleotide drug

delivery, a process currently requiring the use of cationic lipids in cell culture. Our investigations on the syntheses of phosphonoacetate RNA using both TOM and ACE chemistries will be discussed in Chapter 2.

CHAPTER II

SYNTHESIS OF PHOSPHONOACETATE RNA DIMERS USING A 2' SILYL APPROACH

2.1.   Background

As discussed in Chapter 1, previous work has shown that an internucleotide phosphonoacetate linkage can be introduced into 2'-deoxynucleotides (Dellinger, Sheehan et al. 2003). For the preparation of phosphonoacetate linked RNA, the approach begins with a compatible orthogonal protection scheme whereby appropriate 2'-protected ribonucleoside phosphinoamidites were developed for use in the iterative solid phase synthesis cycle. The acid-labile DMT group was selected for protection of the 5'-hydroxyl of the ribonucleoside phosphoramidites for several reasons. First and foremost, this group can be quantitatively removed with anhydrous acid following each round of synthesis (Wu, Ogilvie et al. 1989; Pitsch S 2001). Additionally, the DMT group all ows one to quantify coupling efficiency by monitoring the amount of the DMT cation removed during each deprotection cycle via colormetric and electrochemical conductivity methods (Kaufman, Le et al. 1993). Its use also allowed employing either the TOM or TBDMS group on the 2'-hydroxyl as both were stable to the acidic conditions required for detritylation but removed with fluoride. Because the TOM group has the advantage of typically resulting in higher coupling yields than TBDMS during synthesis, it was selected for investigation. Additionally, it has been shown in the deoxy series that the phosphonoacetate moiety was stable to strong acid. These considerations led us to explore an RNA phosphonoacetate synthesis strategy utilizing nucleoside acetic acid phosphinoamidites having 5'-DMT and 2'-silyl protection.   With this 5'-DMT, 2'-TOM protection scheme in mind, a 1,1-dimethyl-β-cyanoethyl (DMCE) group was selected for the acetate functionality. Previous work with phosphonoacetate DNA synthesis showed the DMCE group can be removed with a 1.5% solution of 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) in anhydrous acetonitrile after oligomer synthesis and before cleavage from the support (Dellinger, Sheehan et al. 2003). This mild reagent would therefore be compatible with the remainder of our synthetic approach.

Also compatible with the above protection scheme was the use of standard nucleobase protecting groups. Standard base protecting groups used in DNA and RNA synthesis are benzoyl (Bz) on N^4 of adenosine, isobutyrl (iBu) on N^2 of guanosine, and acetyl (Ac) on N^4 of cytidine. All of these protecting groups can be removed by treatment with 40% aqueous methylamine (1 hour, 55°C) (Reddy, Hanna et al. 1994).

The solid support was controlled pore glass derivatized with a long chain alkyl amine having a terminal nucleoside attached via a succinyl linker at either the 2'-or 3'-hydroxyl. The vicinal hydroxyl was protected with an acetyl group. Upon treatment with aqueous, or ethanolic methylamine, the RNA oligomer was cleaved from the support with simultaneous removal of the terminal acetyl group, base protecting groups, and the cyanoethyl protecting groups on phosphorus. Following synthesis, and cleavage from support, the TOM group was removed with a 1M solution of tetrabutylammonium fluoride.

2.2.   Results

2.2.1.   Dimethylcyanoethyl Protected Ribouridine Phosphinoamidite Synthesis

Acetic acid, [*bis*(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl ester (**I**) was synthesized according to previously reported protocols (Dellinger, Sheehan et al. 2003) and reacted with 5'-DMT-2'-TOM-uridine (**II**) with one equivalent of dicyanoimidazole (**Figure 2.1**). ^{31}P NMR (**Figure 2.2**) showed peaks at 121 & 126.5 ppm that correspond to two
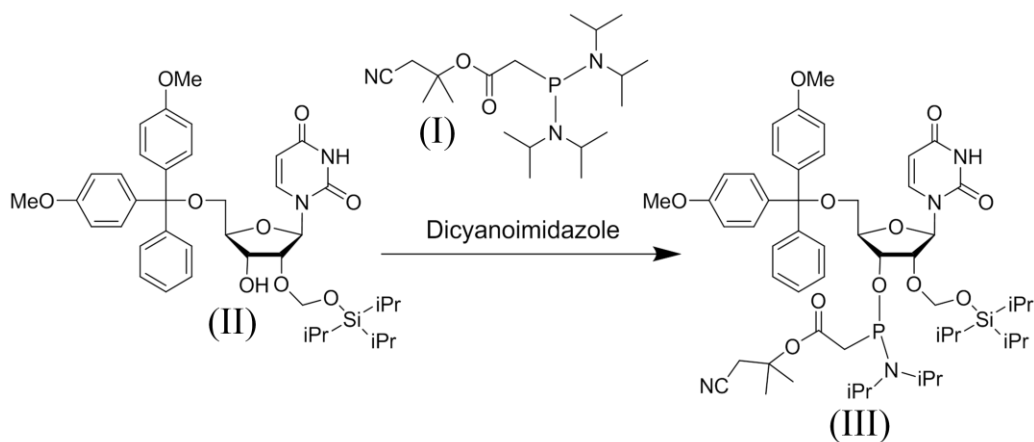
Figure 2.1: **Synthesis of 5'-*O*-DMT-2'-*O*-[(triisopropylsilyl)oxy]methyl-3'-*O*-(diisopropylamino)phosphinoacetic Acid Dimethyl-β-cyanoethylester Uridine (III) (iPr = Isopropyl).**

diastereomers of the expected 3'-phosphinoamidite product (**Figure 2.1**, compound III). This NMR

spectrum also shows a peak at 26 ppm. This corresponds to hydrolysis of the more reactive compound

III.
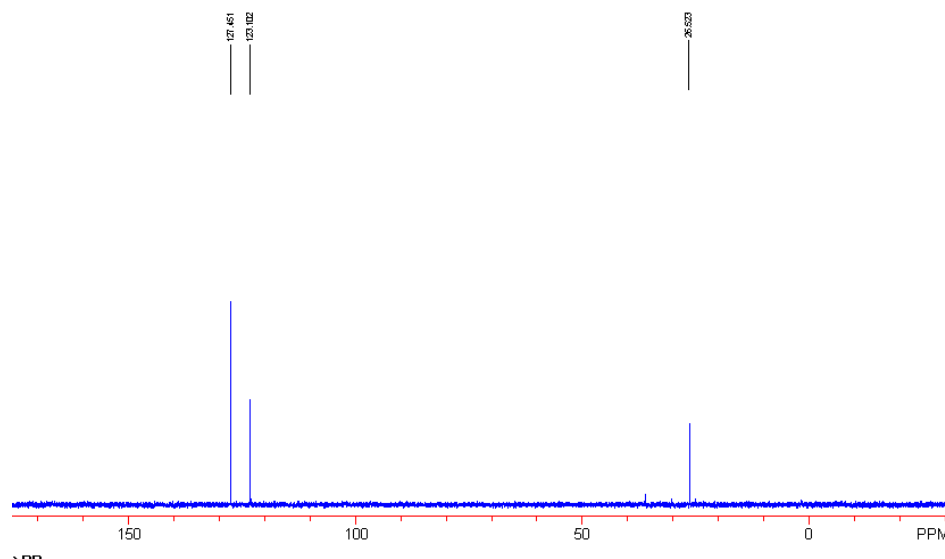
Figure 2.2: **^{31}P NMR of 5'-*O*-DMT-2'-*O*-[(triisopropylsilyl)oxy]methyl-3'-*O*-(diisopropylamino)phosphinoacetic Acid Dimethyl-β-cyanoethylester Uridine (III).**

These chemical shifts agree with known data for the 2'-deoxyphosphinoamidites as previously published (Dellinger, Sheehan et al. 2003). Following silica gel purification, mass spectrometry data were consistent with the desired product.

2.2.2.   Solution Phase Dimer Formation and Characterization

To ensure that the previously described nucleoside phosphinoamidite was reactive and therefore useful in solid phase synthesis, it was reacted with 3'-*O*-acetyl 2'-deoxythymidine in the presence of 1H-tetrazole. 3'-*O*-Acetyl 2'-deoxythymidine was chosen for solution phase synthesis reactions due to commercial availability and acetyl protection at the 3' position. The coupling reaction was monitored using ^{31}P NMR. The ^{31}P NMR chemical shifts moved downfield to 178-180 ppm corresponds to the expected chemical shift for the phosphonite product. After 12 minutes the reaction was complete. Upon oxidation with (1S)-(+)-10-camphorsulfonyl)oxaziridine (CSO), ^{31}P NMR showed a shift upfield to 22-24 ppm as expected for the phosphonoacetate product (IV). This dimer was then purified by reversed phase HPLC and ESI MS confirmed the correct product was formed. Further characterization using ^{31}P NMR

(**Figure 2.3**) revealed two diastereomers at 23.3 & 21.3 ppm, whose chemical shifts were analogous to the

P(V) oxidation state of the phosphonoacetate linkage observed in the deoxy series.
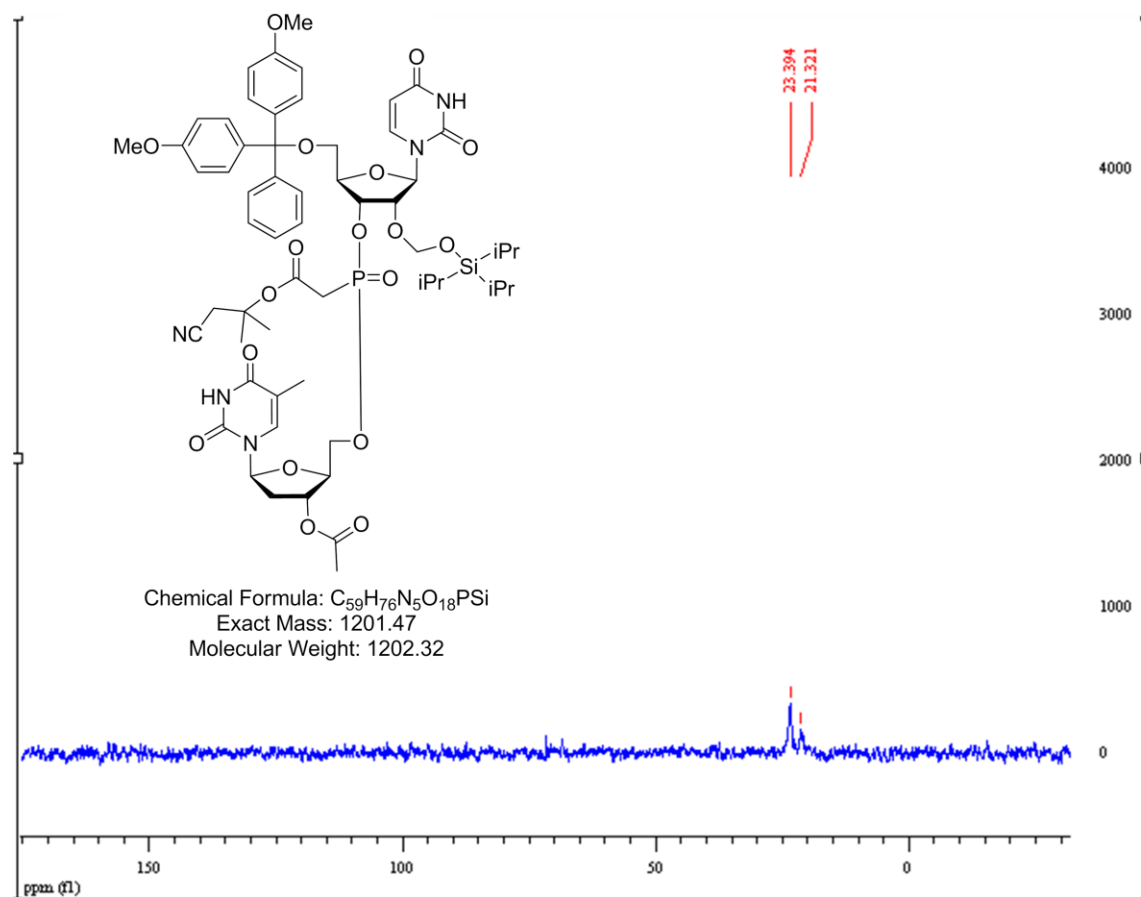
Figure 2.3: ^{31}**P NMR and Structure of Phosphonoacetate Uridine-Thymidylate Dimer (IV) (iPr= Isopropyl).**

2.2.3. **Solid Phase Synthesis of a Uridine Dimer Having a Phosphonoacetate Linkage**

The synthesis cycle used to prepare a uridine dimer having a phosphonoacetate linkage is shown in **Figure 2.4**. There were several modifications relative to the cycle used to prepare DNA. Previous results
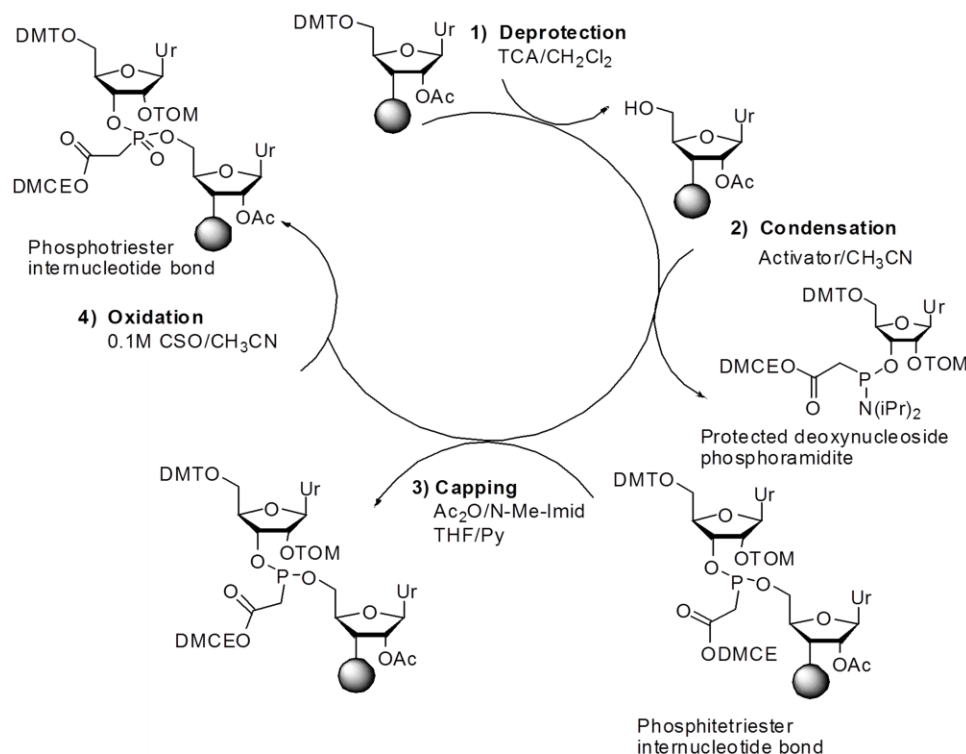
Figure 2.4: Solid Phase Synthesis Cycle Used for Phosphonoacetate Oligoribonucleotide Dimers (Ur =Uridine).

observed during synthesis of phosphonoacetate DNA showed higher quality oligomers when the phosphonite intermediate was oxidized to a phosphonate with (1S)-(+)-10-camphorsulfonyl)oxaziridine (CSO) as opposed to the standard mixture of iodine, pyridine and water (Sheehan et. al.) as used for DNA synthesis. Thus CSO was used for this phosphonoacetate RNA approach. Higher yields of phosphonoacetate DNA oligomers were seen when the capping reagent was changed from *N-*

methylimidazole/pyridine/acetic anhydride (used in standard DNA synthesis) to

dimethylaminopyridine/pyridine/THF/acetic anhydride (Hogrefe, Vaghefi et al. 1993; Dellinger, Sheehan

et al. 2003), thus the capping solution was also changed. For RNA synthesis on polymer supports 4,5-

dicyanoimidazole (DCI) and 5-ethylthio-1H-tetrazole (ETT) were found to be superior to the standard

DNA activator 1H-tetrazole. Therefore, these two activators were chosen in order to optimize coupling

yields.

2.2.4.   Coupling optimization

Coupling yields were determined using (RP HPLC) by quantitating the free uridine and the two

resulting diastereomers . Two activators, 0.25M 5-ethylthio-1H-tetrazole and 0.25M 4,5-

dicyanoimidazole, were studied at a coupling time of 3996 seconds (66 minutes). According to trityl

yields, 0.25 ETT and 0.25 M DCI showed coupling efficiencies of 97% and 89% respectively. Raising the

coupling times past 66 minutes was never examined; however, halving the coupling time lowered the

yields to 83% and 73% for ETT and DCI respectively.

2.2.5.   Ester deprotection and cleavage from support

Following synthesis , the dimethylcyanoethyl ester of the internucleotide phosphonoacetate

linkage was removed while the dimer was still attached to the support (**Figure 2.5**). Removal of this

ester was carried out using a solution of 1.5% DBU in anhydrous acetonitrile (60 minutes). Cleavage

from
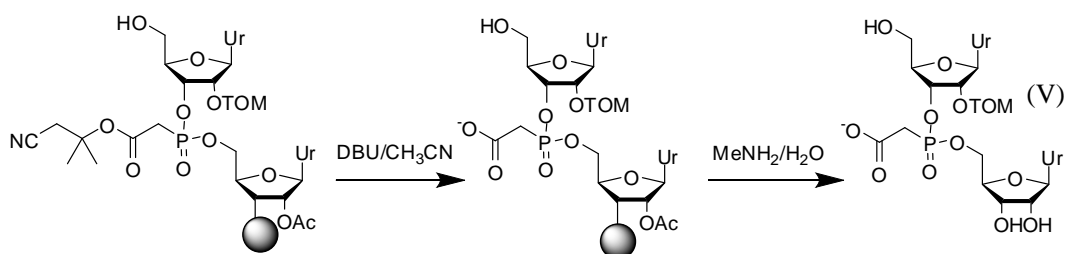
Figure 2.5: Method for the Deprotection of the Dimethylcyanoethyl Group and Cleavage from Support to Form the Phosphonoacetate Uridinyl Dimer (V); DBU = 1,8-Diazabicyclo(5.4.0)undec-7-ene.

the support was then accomplished using 40% methylamine for 1 hour at 55°C. Samples following condensation with either DCI or ETT were then analyzed by RP HPLC in order to further characterize the products as well as to confirm coupling yields using the trityl cation assay. Integration of the HPLC chromatographs allowed for quantitation of free uridine compared to product dimer (**Figure 2.6**).
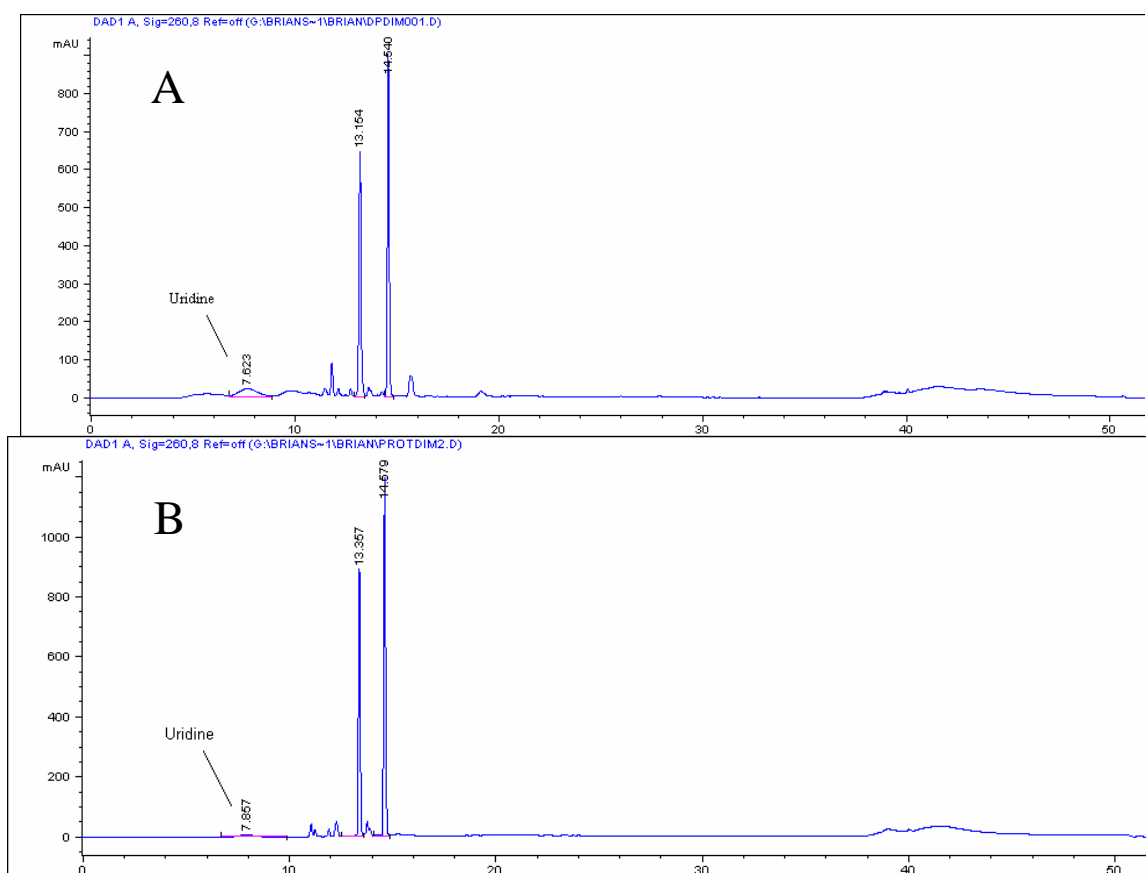
Figure 2.6: RP HPLC Chromatograph of Phosphonoacetate Dimers (Showing Two Diastereomers). Panel A: Dimer Synthesized Using 0.25M DCI as an Activator; Panel B: Dimer Synthesized Using 0.25M ETT as an Activator.

Using this method, the samples prepared using ETT and DCI had calculated coupling yields of 96% and 85% respectively. ^{31}P NMR of the dimer showed peaks at 29.5 and 28.2 ppm which correspond to the diastereomers (**Figure 2.7**). Negative mode ESI MS gave the correct mass at 760.9 m/z.
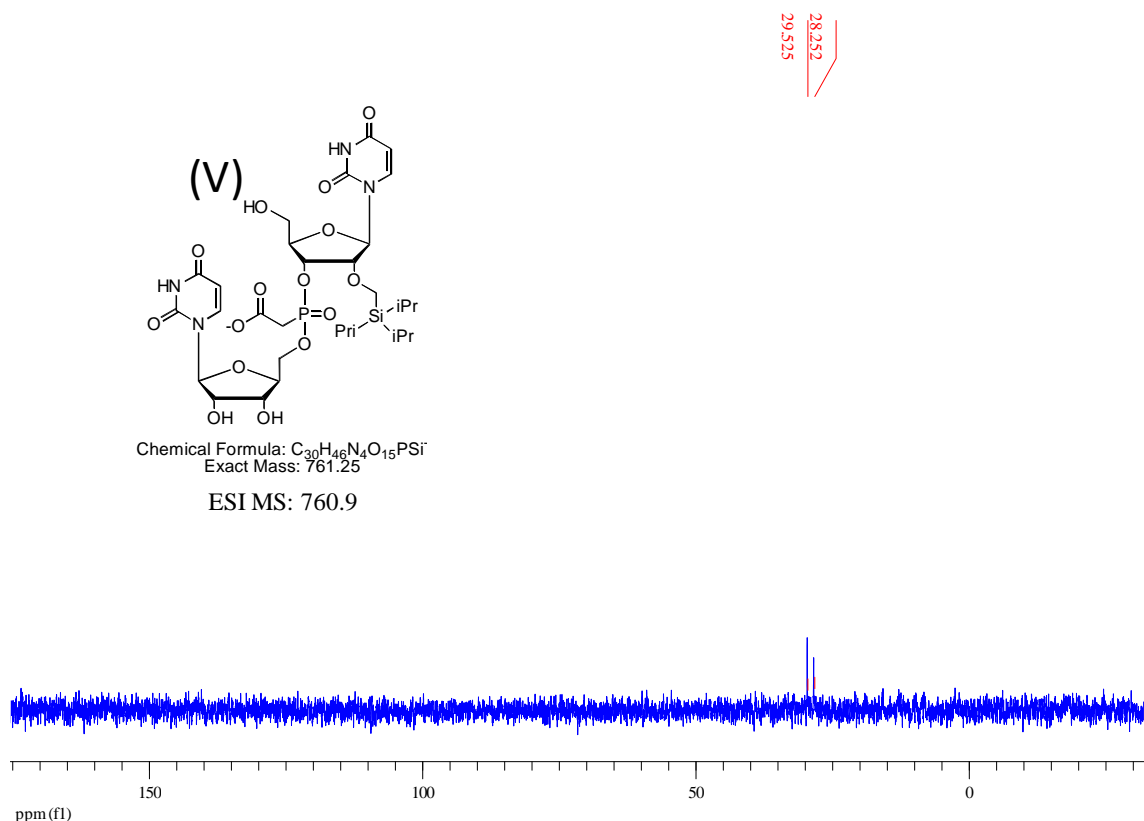
Figure 2.7: ^{31}P NMR Spectrum of the Phosphonacetate Dimer (V) after Ester Deprotection and Cleavage from Support. Peaks Were Observed at 29.5 & 28.2 ppm.

2.2.6.   2' TOM deprotection

The final step in the synthesis of a phosphonoacetate uridinylate dimer using the TOM protection strategy was removal of the TOM group. According to the literature, this silyl blocking group was removed with 1.0M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (12 hours.) Removal of the silyl group was a two step process. Initially, removal of the silyl group generates a 2'-hemi-acetal.

Removal of the hemiacetal proceeded by treatment with a mild, basic buffer, which releases

formaldehyde, and generates the free 2'-hydroxyl (Pitsch S 2001). This procedure was repeated on the

phosphonoacetate uridinyl dimer. After 12 hours, ^{31}P NMR data were recorded on the crude mixture and

showed two peaks at 16.9 and 16.7 ppm (**Figure 2.8**).
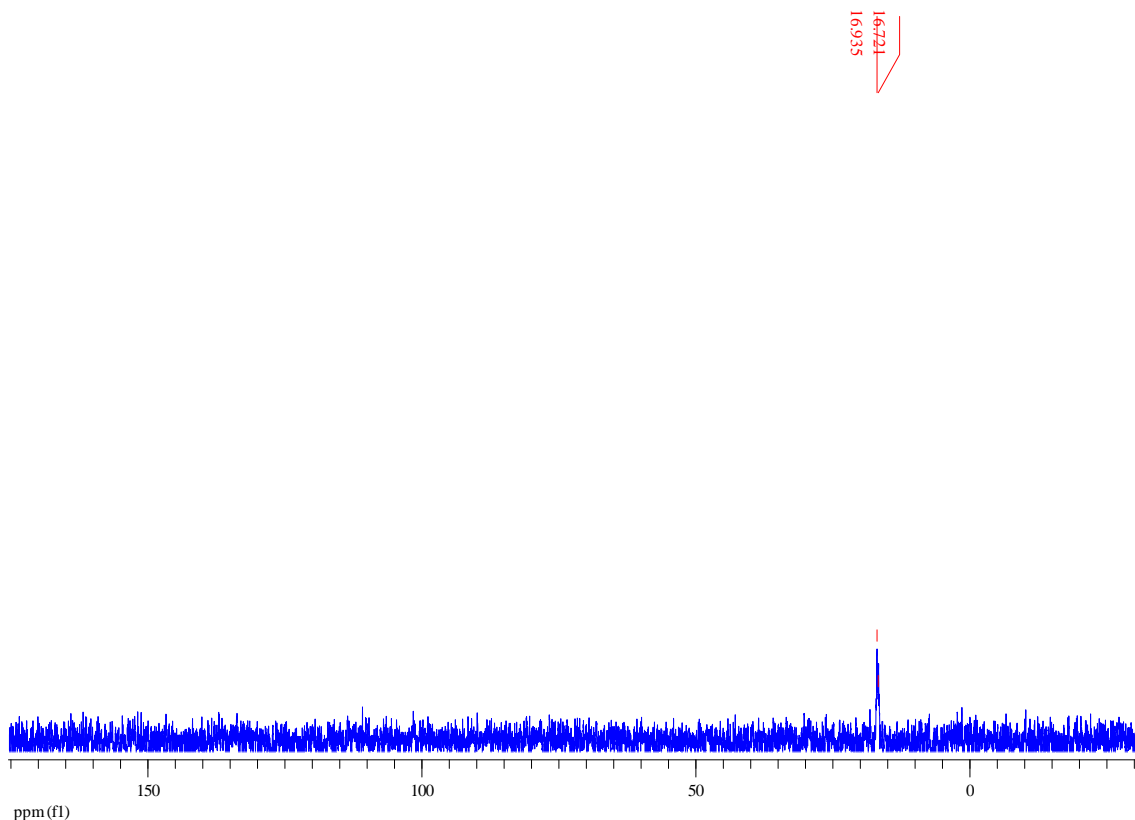
Figure 2.8: **^{31}P NMR Spectrum of Phosphonoacetate Uridine Dimer (V) after Treatment with TBAF for 12 Hours Shows Peaks at 16.9 & 16.7 ppm.**

RP-HPLC showed a change in retention time, with an apparent increase in the amount of free uridine

(**Figure 2.9**). These data, suggests that the phosphonoacetate linkage was unstable to treatment of with

TBAF.
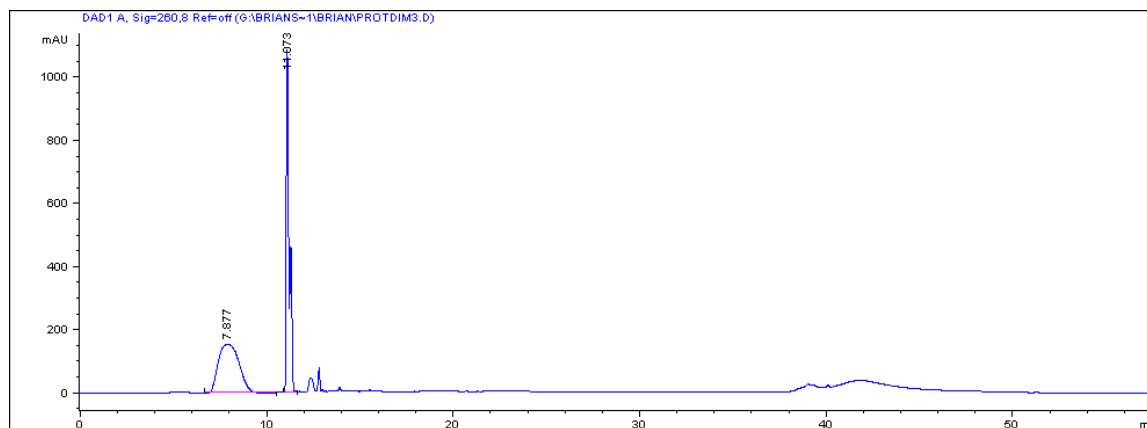
Figure 2.9: RP HPLC Chromatograph of Phosphonoacetate Uridine Dimer (V) after Treatment with TBAF for 12 Hours.

2.3.   Discussion

The synthesis of 5'-*O*-DMT-2'-*O*-TOM protected uridine phosphinoamidite proceeded smoothly and was shown to be compatible with standard phosphitylation procedures. The phosphitylation of the TOM protected ribonucleoside did proceed far slower than seen with the analogous reaction on a 2'-deoxynucleoside substrate (Dellinger, Sheehan et al. 2003). This result was expected, however, as the added steric bulk and proximity of the protecting group on the 2'-hydroxyl may impede the progress of the reaction.

During solution phase synthesis of the ribonucleoside phosphinoamidite with 3'-*O*-acetyl thymidine, the ribonucleotide synthon proved to be reactive toward formation of the phosphonite triester intermediate and also oxidation using CSO.   Analysis of ^{31}P NMR chemical shifts to literature values observed in the DNA series showed that the results were comparable. Identity of the desired dinucleotide was confirmed by ESI-MS.

All the above results indicated that a 2'-*O*-TOM protected phosphinoamidite would be sufficiently reactive for use in solid phase synthesis. Furthermore, the oxidation, ester deprotection and cleavage conditions appeared to be compatible for use in synthesizing phosphonoacetate RNA using the described phosphinoamidite. The solid phase synthesis of a phosphonoacetate uridinyl dimer, TOM-protected at the 2- hydroxyl was successfully achieved with 95% yield and few by-products as judged by RP-HPLC. The required coupling time was considerably higher than the standard RNA synthesis cycles. NMR and mass spectral data confirmed the identity of the dimer. Upon treatment of the phosphonoacetate ribonucleotide dimer with TBAF, it was seen to decompose quantitatively to what appeared to be uridine and two products. The identity of the decomposition products were not confirmed in these experiments; however, data presented in Chapter 3 suggest these are most likely a phosphonoacetate monoester and free nucleoside, the result of internucleotide bond cleavage. TBAF is known to be quite basic as it has been shown to often contain significant amounts of tributylamine (Pilcher and DeShong 1996), so it is possible these conditions caused alkali promoted transesterification leading to cleavage of the phosphodiester bond.

Materials and Methods

2.3.1. General Procedures

Unless otherwise stated, all materials were obtained from commercial suppliers and used without further purification. 5'-*O*-DMT-3'-*O*-TOM-ribouridine was a generous donation from Glen Research Corp. Silica Gel as used for preparative column purification (230 X 400 mesh) was obtained from Sorbent Technologies. Thin layer chromatography was performed using aluminum backed silica 60 F_{254} plates. Reversed phase HPLC was performed on an Agilent 1100 series HPLC instrument using a Hypersil ODS 4.0 X 250mm C-18 column. ^{31}P NMR data were recorded on a Varian 400 MHz

spectrometer. ESI MS data were obtained using an ABI Pulsar Q-Star Q-TOF spectrometer. FAB MS

data was performed by the University of Colorado Central Analytical Laboratories.

2.3.2.   Preparation of Acetic Acid, [*Bis*(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl Ester(I)

In a two neck 1L round bottom flask, diethyl ether (200 ml) was brought to reflux, granular zinc

(6.54 g, 100 mmol) was added, and the mixture stirred for 20 minutes. 1,1-Dimethyl-2-cyanoethyl acetyl

bromide (8.80 g, 40 mmol) and *bis*(*N*,*N*-diisopropylamino)chlorophosphine (10.67 g, 40 mmol) were

dissolved in anhydrous tetrahydrofuran (400 ml) and 200 ml of this solution was added to the refluxing

ether. The combined solution was brought to reflux and allowed to react for 10 min. before adding the

remaining 200 ml. The resulting reaction mixture was allowed to reflux for 2 hours, cooled to room

temperature, and concentrated to a thick, orange oil *in vacuo*. The residue was triturated with anyhydrous

hexanes (2 x 500 mL). Pure product (10.10g, 68% yield) was recovered from the hexanes and

characterized by phosphorus NMR and mass spectrometry. ^{31}P NMR: δ 48.2 ppm. ESI MS: 371.3 *m/e*.

2.3.3.   Synthesis of 5'-*O*-DMT-2'-*O*-[(triisopropylsilyl)oxy]methyl-3'-*O*-(diisopropylamino)phosphinoacetic acid dimethyl-β-cyanoethylester uridine (II)

5'-*O*-DMT-2'-O-[(triisopropylsilyl)oxy]methyl ribouridine (5 mmol) was dissolved in

dichloromethane (200 mL). Acetic acid, [*bis*(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-

cyanoethyl ester (5.00 mmol) and dicyanoimidazole (5.00 mmol) were added and the reaction mixture

stirred at room temperature for 10h. The reaction was neutralized with *N,N,N',N'*-

tetramethylethylenediamine (5.50 mmol), concentrated in vacuo and the crude reaction mixture loaded

directly onto a silica column without further workup. Product was purified from this column by elution

with a gradient of 10-60% ethyl acetate/hexanes/0.1% TEA. Pure product (4.86 g, 96 % yield) was

isolated as a white crystalline solid. ^{31}P NMR: δ121.5 & 126.5ppm. FAB MS: 1002 m/z.

2.3.4. Phosphonoacetate Uridine-Thymidylate Dimer Synthesis and Purification

In a dry one dram vial, 5'-*O*-DMT-2'-*O*-[(triisopropylsilyl)oxy]methyl-3'-*O*-(diisopropylamino)phosphinoacetic acid 1,1-dimethyl-β-cyanoethylester uridine (20mg, 0.020 mmol) was dissolved in anhydrous acetonitrile (1.0 mL). 3'-*O*-Acetyl-2'-deoxythymidine (5.67mg, 0.20 mmol) and dicyanoimidazole (21.2mg, 0.179 mmol) were added and the reaction mixture shaken on a stationary shaker. The reaction was monitored by ^{31}P NMR and upon completion (12 min), (1S)-(+)-10-camphorsulfonyl)oxaziridine (13.7mg, 0.60 mmol) was added and ^{31}P NMR data was again recorded. One mL of this mixture was injected directly onto the HPLC. Product was eluted with a gradient of 15-85% acetonitrile/water in 65 minutes. ^{31}P NMR: δ 23.3 & 21.3ppm. ESI MS: 1187 [M+H] & 885 [M-trityl].

2.3.5. Synthesis of phosphonoacetate oligoribonucleotide dimers

All DNA/RNA synthesis reagents including activators, solid support columns, and capping reagents were purchased from Glen Research (Sterling, VA). The solid phase synthesis of phosphonoacetate dimer was performed on an ABI 394 DNA/RNA Synthesizer acquired from Applied Biosystems (Foster City, CA). All solid phase syntheses were performed on a one micromole scale and the cycle was adapted from a standard phosphonoacetate DNA synthesis cycle with the coupling time increased to 3996 secconds (1 hour, 6 min.). Phosphinoamidite monomers (0.1M in anhydrous acetonitrile), capping solution B (0.625% w/v *N,N*-dimethylaminopyridine in anhydrous pyridine), oxidant (0.1M in anhydrous acetonitrile) and activator (0.25M ETT or DCI in anhydrous acetonitrile) were all prepared immediately prior to use.

After synthesis, CPG columns were washed with anhydrous acetonitrile and dried with argon. Anhydrous DBU (stored over molecular sieves) was diluted to 1.5% in anhydrous acetonitrile and applied to the column between two, one mL syringes and allowed to react for 60 minutes by pushing the solution occasionally back and forth through the column. The CPG was washed thoroughly with acetonitrile, dried extensively with argon, and placed in a one dram vial. Methylamine (40% aq., 1.5 mL) was added and the vial sealed with a teflon lined screw cap. The vial was placed in a 55°C heating block and allowed to react for 1 hour. Following the cleavage reaction, the vial was cooled on ice, the CPG was removed by filtration and the supernatant evaporated to near dryness *in vacuo*.

2.3.6.   RP HPLC analysis of phosphonoacetate oligoribonucleotide dimers

Crude dimers were analyzed on an Agilent 1100 series HPLC instrument using a Hypersil ODS 4.0 X 250mm C-18 column. Eluents were: (A), 100mM triethylammonium acetate in water, pH 7.5; (B), acetonitrile. The eluent gradient was 0-30% B in 32 minutes at a flow rate of 1.0 mL/min while observing at wavelength 260nm. Relative areas of each peak were determined by integration.

CHAPTER III

SOLID PHASE SYNTHESIS AND CHARACTERIZATION OF PHOSPHONOACETATE OLIGORIBONUCLEOTIDES SYNTHESIZED VIA 2'-ORTHOESTER AND 2'-ACETAL PROTECTED RIBONUCLEOSIDE 3'-PHOSPHINOAMIDITES

3.1.   Background

As discussed in the previous chapter, the conditions required to remove the TOM group from the 2' hydroxyl were incompatible with the phosphonoacetate internucleotide linkage. An alternate strategy for the solid phase synthesis of this analog was to switch the orthoganality to a strategy where the 5'-protecting group was acid stable and the 2'-protecting group was acid labile. More specifically, it is possible to use a 5'-fluoride labile protecting group in combination with a 2'-acid labile orthoester on the phosphinoamidite monomers. This strategy will lead to a protected phosphonoacetate oligomer having a 2'-orthoester protecting group. The phosphonoacetate linkage is stable to very acidic conditions(pH 1-10 at 95°C) while incorporated into an oligodeoxynucleotide (Sheehan 2001). Therefore, an acid labile protecting group such as an orthoester on the 2'-hydroxyl would be a logical approach to the solid phase synthesis of phosphonoacetate RNA.

Many acid labile protecting groups have been examined for use on the 2'-hydroxyl. RNA oligomers are very unstable in an acidic medium due to acid catalyzed transesterification (Oivanen, Kuusela et al. 1998). Therefore it is necessary to remove the 2'-protecting group rapidly and in a medium above pH 3.0 (Li and Breaker 1999). Because the stability of phosphonoacetate RNA oligomers in acidic and basic media was unknown, it was assumed that its reactivity would be somewhat similar to natural RNA because of the possibility for similar transesterification reactions due to the proximity of the 2'-

hydroxyl. Therefore, it was presumed that phosphonoacetate RNA would be vulnerable above an

approximate pH of 9.5 and below pH 3.0. As discussed in previous chapters, the steric bulk of the

protecting group is also a concern because of the close proximity of the 2'-protecting group to the

reaction site at the 3'-hydroxyl. This concern is magnified when attempting to synthesize

phosphonoacetate RNA because the phosphinoamidites used during synthesis contain the very bulky

dimethylcyanoethyl group as opposed to cyanoethyl or methyl protection on phosphate. The large

dimethylcyanoethyl group was originally used to sterically prevent nucleopilic attack at the ester

functionality and at the phosphorous atom (Sheehan 2001).

The two acid labile protecting groups examined in this research were the [*bis*(2-acetoxyethoxy)

methyl] (ACE) and the 4-methoxy-2H-tetrahydropyran-2-yl (MTHP) (**Figure 3.1**).
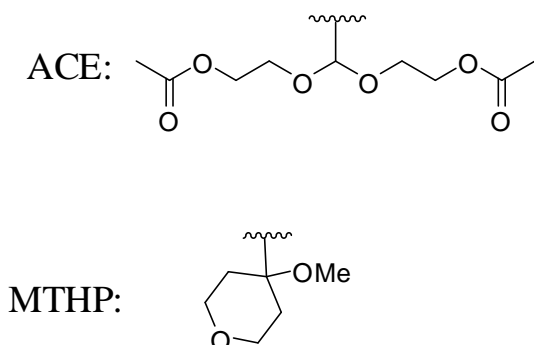
Figure 3.1: Structures of the [*Bis*(2-acetoxyethoxy)methyl] (ACE) and the 4-Methoxy-2*H*-tetrahydropyran-2-yl (MTHP) Protecting Groups.

The ACE group is an orthoester developed by Scaringe et al (Scaringe, Wincott et al. 1998). It is

very rapidly removed under mildly acidic conditions such as 100mM acetic acid, pH 3.8, in less than an

hour. The ACE group has been used very successfully in combination with 5'-siloxyl protection to

synthesize oligoribonucleotides in very high yield and purity. Despite its large size overall, it lacks the

steric bulk local to the 3'-hydroxyl that, for example, the TBDMS group carries with it. This is because

the TBDMS group bears two methyl groups and a tertiary butyl group on the silyl atom directly attached

to 2'-oxygen whereas the orthoester consists of a carbon atom bearing an oxygen and two ethyl groups, representing less atomic volume at the 2'-hydroxyl. Scaringe and co-workers report coupling yields in excess of 99% per cycle using this chemistry to synthesize RNA.

The MTHP group requires a treatment of 80% acetic acid for over two hours for complete removal (Reese and Skone 1985). Such acidic conditions result in phosphate migration as well as strand cleavage due to acid catalyzed transesterification in natural RNA; however, it was unknown if the phosphonoacetate linkage would behave in the same way. Thus the MTHP group was chosen as a possible candidate for 2'-hydroxyl protection.

Employing 2'-acid labile protection requires using non-acidic conditions for the removal of the 5'-protecting group. Recent work by Scaringe et al. generated [(benzhydroxy)*bis*(trimethylsilyloxy)]silyloxyl (BzH) as a 5'-fluoride labile protecting group (**Figure 3.2**).
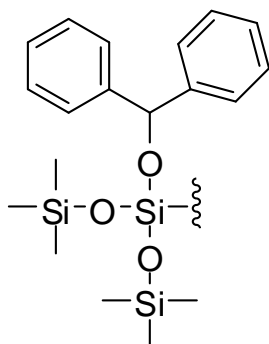
Figure 3.2: Structure of the [(Benzhydroxy)*bis*(trimethylsilyloxy)]silyloxyl Protecting Group (BzH).

BzH, which is labile to fluoride ion as opposed to acid, requires only 35 seconds for complete removal following each round of synthesis. These conditions would also allow for proper orthogonality with both the ACE and the MTHP group as both ACE and MTHP groups are stable to fluoride ion.

Post-synthesis, oligomers bearing either the MTHP or ACE protecting groups were cleaved from support with aqueous methylamine or concentrated ammonia. In the case of oligomers protected with the ACE group, this methylamine treatment also removes the acetyl groups on the orthoester and thus converts it to the *bis*(2-hyroxyethoxy)methyl orthoester. This conversion renders the 2'-protecting group 10 times more acid labile than the ACE orthoester and soluble in aqueous media. Following cleavage from support, both the MTHP and the ACE protecting groups can be hydrolyzed from the 2'-hydroxyl with aqueous acid.

3.2. Results

3.2.1. 2'-ACE and 2'-MTHP Protected Phosphinoamidites

Synthesis of the protected ribonucleosides was accomplished using the scheme shown in **Figure 3.3**.
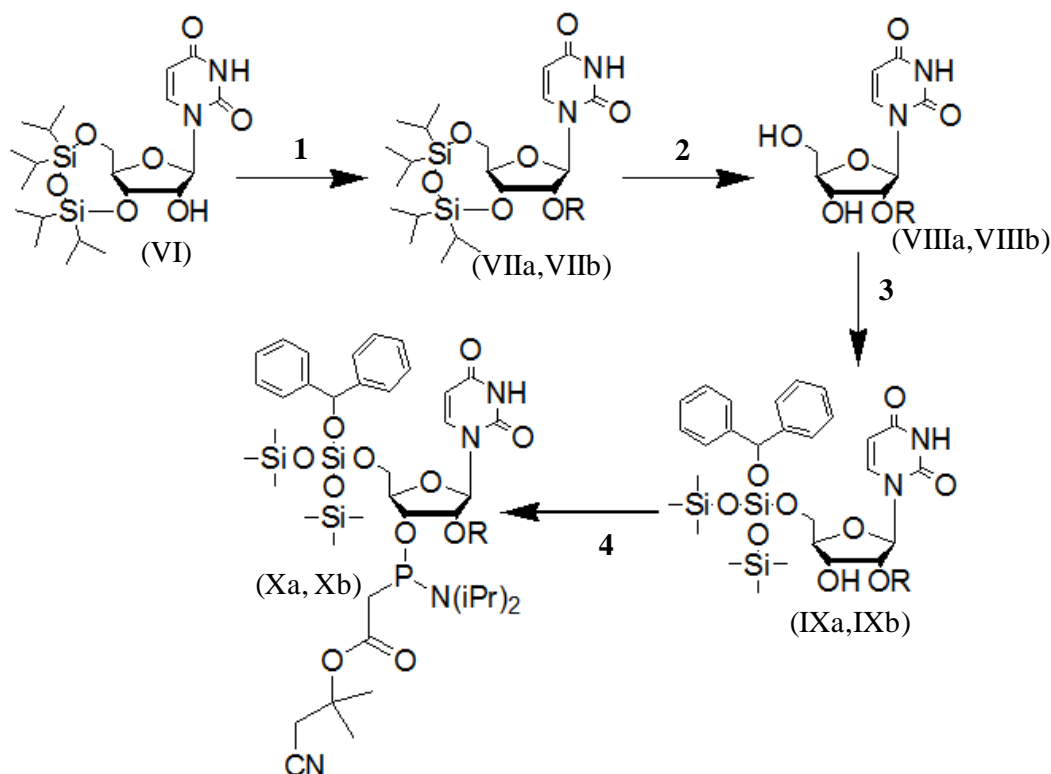
Figure 3.3: Synthetic Scheme Used to Synthesize Protected Phosphinoamidites (Xa & Xb); R = ACE(a Compounds) or MTHP (b Compounds). 1: 4-Methoxy-3,6-dihydro-2H-pyran or *tris*(2-acetoxyethoxy)orthoformate, H⁺. 2: TEMED/HF; 3: [(Benzhydroxy)*bis*(trimethylsilyloxy)]silylchloride, Diisopropylamine; 4: [*Bis*(N,N-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl Ester, DCI.

Use of the commercially available 5',3'-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl) (TIPS) protected uridine (**VI**) allowed for selective protection of the 2'-hydroxyl under a variety of conditions as the TIPS group was stable to mild acid, mild base and was easily removed with fluoride ion. Once the 2'-hydroxyl was protected with either MTHP or ACE (**VIIa** & **VIIb**), TIPS was removed (**VIIIa** & **VIIIb**), and the 5'-hydroxyl can be protected (**IXa** & **IXb**). Regioselective protection of the 5'-hydroxyl was accomplished by taking advantage of the fact that the 5'-hydroxyl was a primary alcohol, whereas the 3'-hydroxyl was a secondary alcohol. Also, the BzH group was designed to be a bulky reagent so that it will react preferentially with the 5'-hydroxyl and not the sterically crowded 3'-position. As a result, 5'-silylation consistently results in yields in excess of 85% after purification. With the 5'- and 2'-hydroxyls

protected, both 2'-MTHP or ACE protected nucleosides can now be phosphitylated to the corresponding phosphinoamidites (compounds **Xa** and **Xb**). The phosphitylation yielded a ^{31}P spectrum very similar to that of the TOM phosphinoamidites. Each showed two peaks in the range of 125-118 ppm (**Figure 3.4 & 3.5**). Mass spectrometry confirmed their identity.
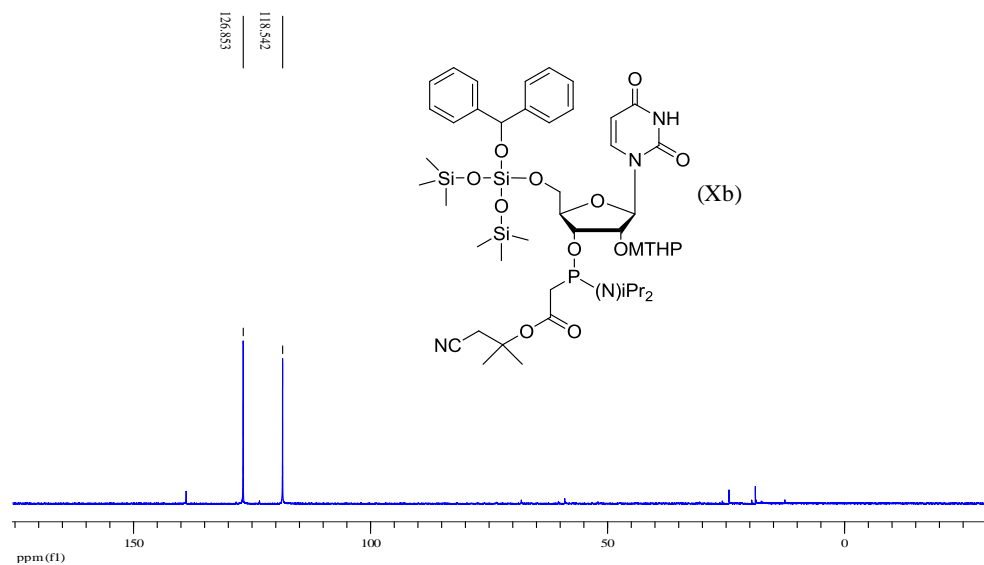
Figure 3.4: ^{31}P NMR of 5'-*O*-BzH-2'-*O*-MTHP- Uridine Phosphinoamidite (Xb).
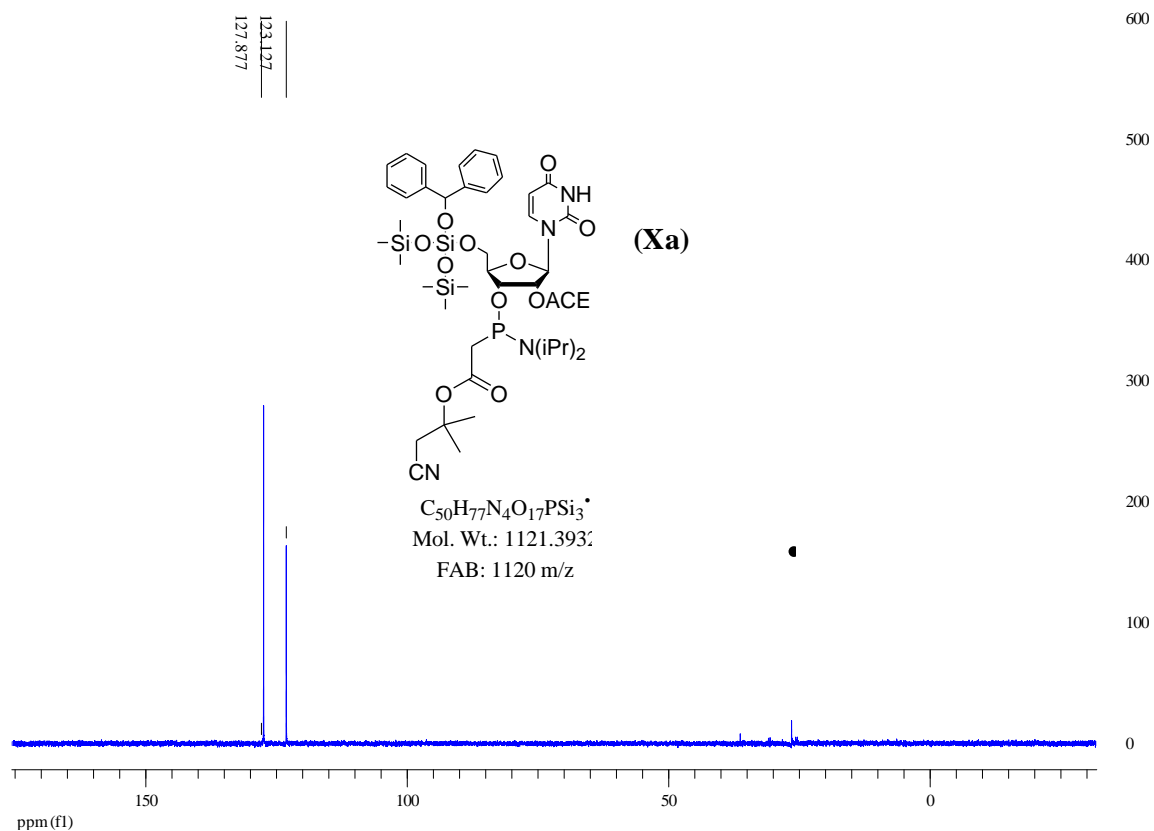
Figure 3.5: ^{31}P NMR of 5'-*O*-BzH-2'-*O*-ACE Protected Uridine Phosphinoamidite (Xa).

3.2.2. Solution Phase Uridine-Thymidylate Dimer Formation and Characterization

To further characterize 5'-*O*-[(benzhydroxy)*bis*(trimethylsilyloxy)]silyloxyl-2'-*O*-[*bis*(2-acetoxyethoxy)methyl]-3'-*O*-(diisopropylamino)phosphinoacetic acid dimethyl-β-cyanoethyl ester ribouridine (**Xa**), it was reacted with 3'-acetylthymdine in an NMR tube using ethylthiotetrazole as an activator. After, 10 minutes the coupling reaction was complete, as seen by phosphorus NMR. After addition of (1S)-(+)-10-camphorsulfonyl)oxaziridine (CSO) to the reaction, two peaks at 23.4 & 21.1 ppm, corresponding to the correct oxidation product of phosphonoacetate DNA, were observed (**Figure 3.6**) (Sheehan 2001).
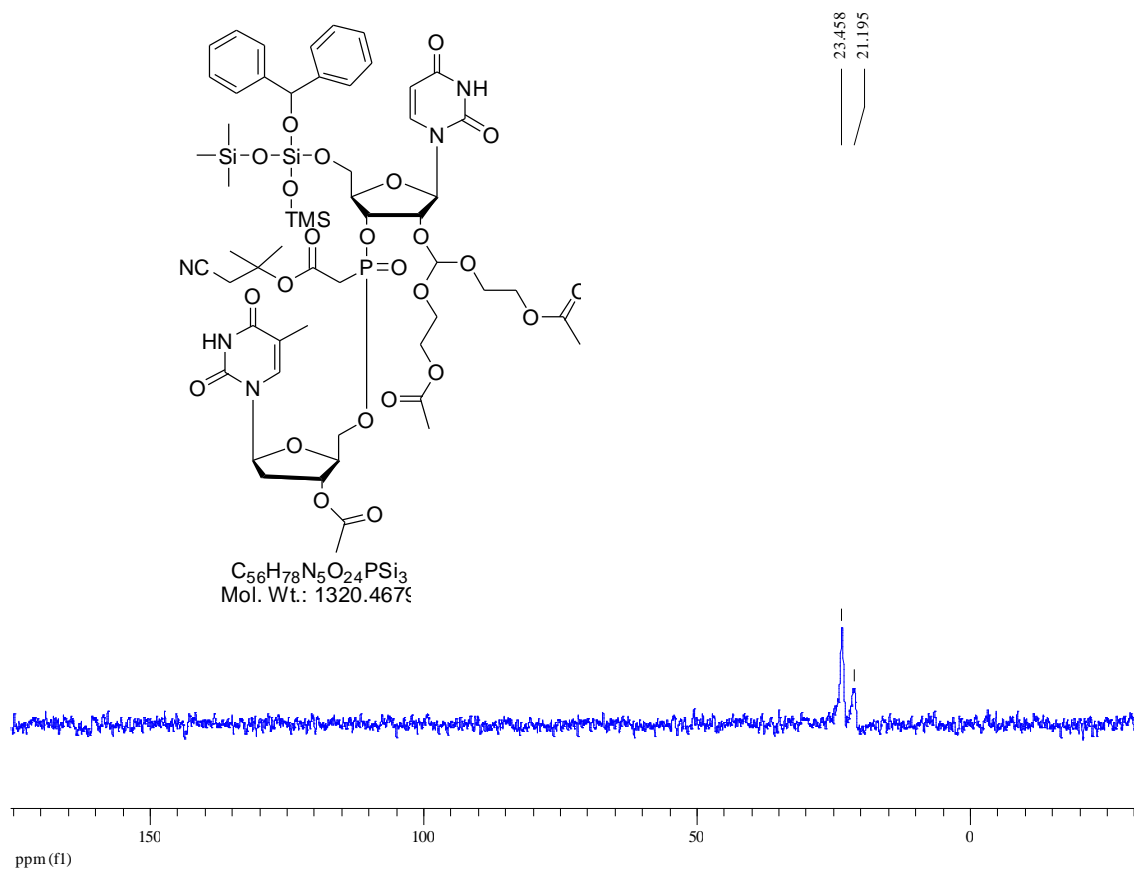
Figure 3.6: ^{31}P NMR of 2'-O-ACE Protected Phosphosphonoacetate Uridine-Thymidylate Dimer.
This dimer was purified by preparatory HPLC (**Figure 3.7)** and characterized by ESI MS (**Figure 3.8**).
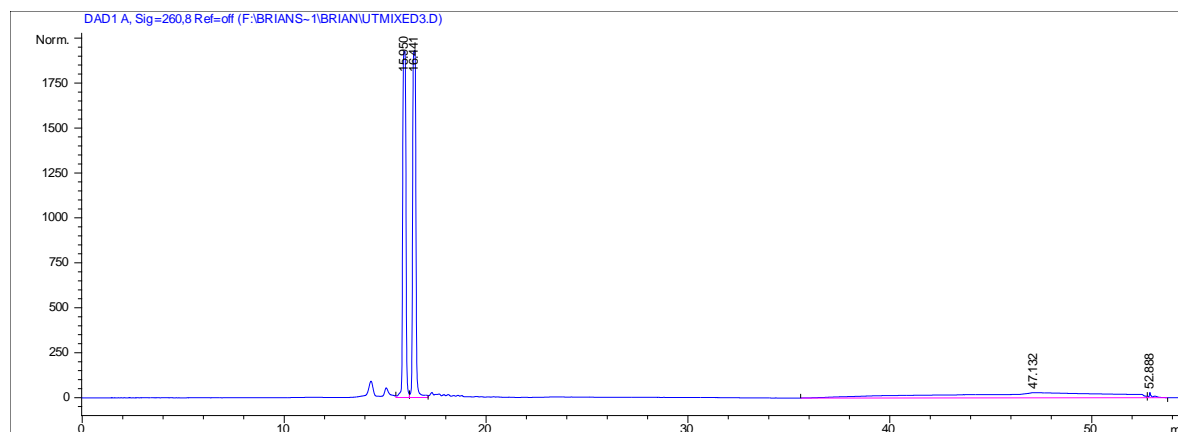
Figure 3.7: Reverse phase HPLC Chromatograph of a Purified, Fully Protected 2'-ACE Phosphphosphonoacetate Uridine-Thymidylate Dimer.
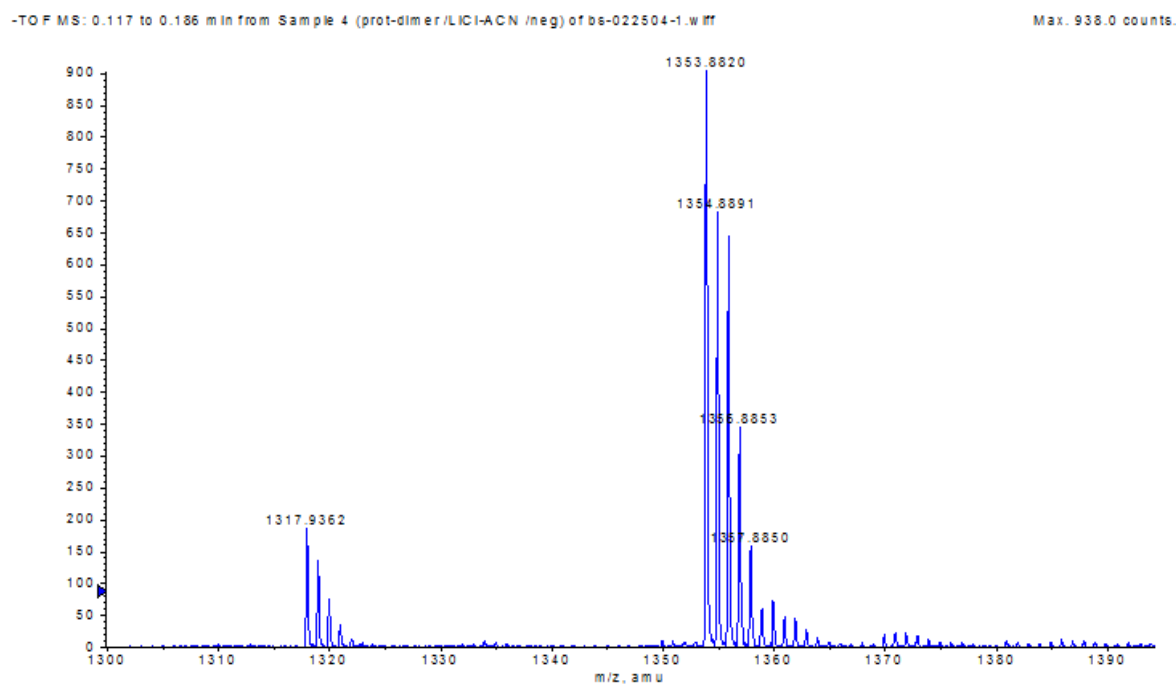
Figure 3.8: Negative Mode ESI Mass Spectrum of a Purified, 2'-ACE Phosphonoacetate Uridine-Thymidylate Dimer; [M-H]⁻ at 1317.9 and [M+Cl]⁻ at 1353.8.

With the phosphorus NMR profile of a phosphonoacetate dimer correctly characterized, the 2'-MTHP protected synthon was never used to make a dimer in solution but instead used immediately in solid phase synthesis.

3.2.3. Solid Phase Synthesis Cycle

The cycle used for solid phase phosphonoacetate RNA synthesis is illustrated in **Figure 3.9**.
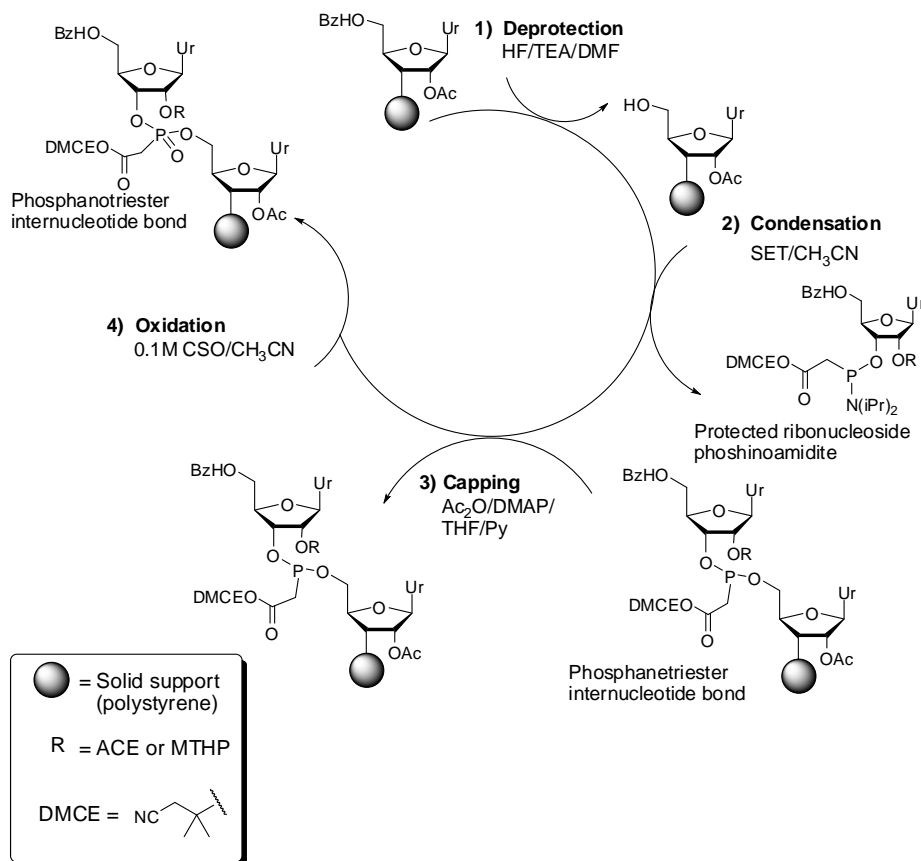
Figure 3.9: Solid Phase Synthesis Cycle Used in the Synthesis of Phosphonoacetate RNA Oligomers Utilizing a 2'-Acid Labile Protection Strategy; Ur = uridine.

This cycle is a direct adaptation of a strategy published by previous researchers in the Caruthers

laboratory utilizing fluoride labile protecting groups on the 5'-hydroxyl (Scaringe 1996). A polystyrene-

linked, 5'- BZH-2'-acetyl protected ribonucleoside was first deprotected with 4% (v/v) solution of

hydrofluoric acid in dimethylformamide and the oligomer elongated from the 5'-hydroxyl. The coupling

step consisted of adding a 0.1M solution of a protected 3'-ribonucleoside phosphinoamidite and 0.25M 5-ethylthio-1H-tetrazole (ETT) in anhydrous acetonitrile. Based upon previous experiments on the formation of phosphonoacetate RNA dimers in solution, a reaction time of 66 minutes was used (four coupling times of 999 seconds, the maximum time point of an ABI 394 synthesizer). Unreacted 5'-hydroxyls were capped with a solution of dimethylaminopyridine/pyridine/THF/acetic anhydride. The acetic acid phosphonite was converted to the P(V) phosphonate using a 0.1M solution of CSO in anhydrous acetonitrile for 180 seconds. The 5' siloxyl group was removed from the growing chain using a 35 second treatment with 4% (v/v) solution of hydrofluoric acid in dimethylformamide. This cycle was then repeated several times in order to generate an oligoribonucleotide.

The dimethylcyanoethyl ester on the phosphonate backbone was removed with a 1.5% (v/v) solution of DBU (1,8-Diazabicyclo(5.4.0)undec-7-ene) in acetonitrile for 1 hour while the oligomer was still support-bound. The support was then treated with a 40% methylamine solution for 40 minutes, which removed the 2'-protected phosphonoacetate oligoribonucleotide from the support.

3.2.4.   Synthesis and Analysis of Poly Uridinyl Phosphonoacetate Oligoribonucleotides

To quantitate the coupling efficiency of the ACE protected monomer versus the MTHP protected monomer, ion exchange high performance liquid chromatography (IE HPLC) was used. The inclusion of a capping step in the synthesis cycle allowed for the quantitation of failure sequences by IE HPLC and in turn, coupling efficiency. To this end, two poly-uridinyl sequences, both 7 bases in length, representing 6 coupling events, were synthesized using the synthesis cycle described above. The DMCE group from the phosphonoacetate ester was deprotected from the two 7mers using a solution of 1.5% DBU in acetonitrile, the oligomers cleaved from support using 40% methylamine and the crude material analyzed by IE HPLC. According to the chromatograms in **Figure 3.10**, the MTHP protected monomer had a coupling yield of 79% whereas the ACE protected monomer coupled at 94% efficiency.
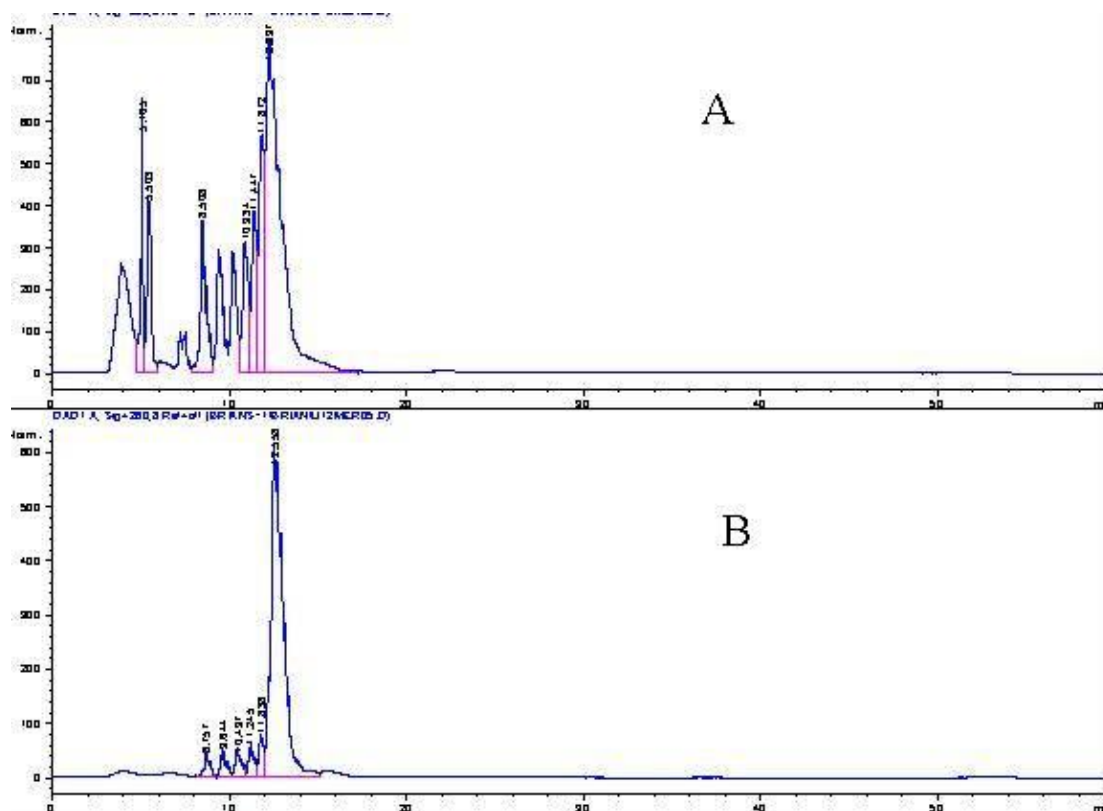
Figure 3.10: Ion Exchange HPLC Chromatograms of Crude, 2'-Protected Phosphonoacetate Uridine 7mers. A: 2' MTHP Protected Oligomer; B: 2' ACE Protected Oligomer.

The chromatograms in **Figure 3.10** show very broad peaks due to each phosphonoacetate linkage having two diastereomers. Early eluting peaks (less than 11 minutes) are indicative of failure sequences resulting from incomplete coupling. ^{31}P NMR of the oligomers showed a range of peaks in the area of 30-28 ppm which was the expected chemical shift of the phosphonoacetate linkage.

The deprotection of the 2'-hydroxyl of crude product was characterized by reverse phase HPLC. In order to remove the 2-hydroxyl protecting groups, the ACE protected 7mer was treated with 100mM acetic acid, pH 3.8, for 30 minutes at 55°C and the MTHP protected 7mer with 80% acetic acid for 1 hour at 55°C. The individual samples were immediately analyzed by HPLC. **Figure**s **3.11** and **3.12** show RP HPLC chromatograms of the ACE and the MTHP protected 7mers both before and after deprotection.
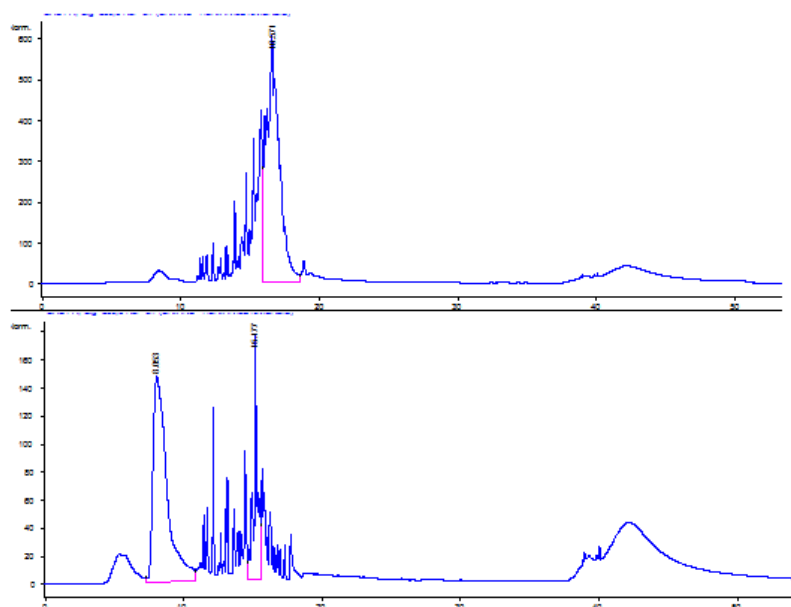
Figure 3.11: Reversed phase HPLC Chromatograms of Crude Phosphonoacetate Uridine 7mers Synthesized with 2' MTHP Protection; TOP: Before 2'-Deprotection; BOTTOM: After 2'-Deprotection
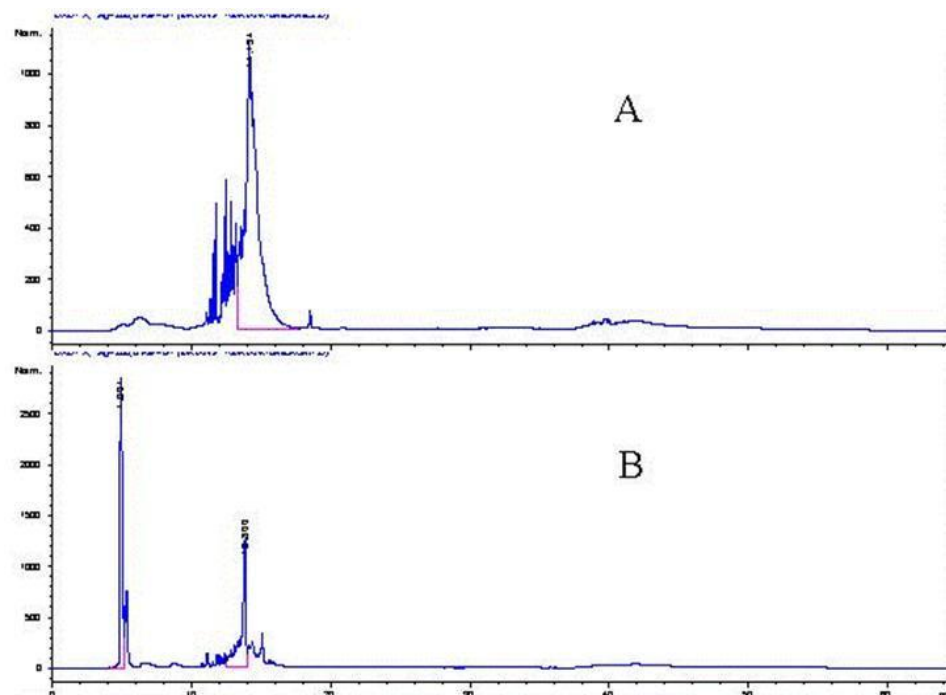
Figure 3.12: Reversed Phase HPLC Chromatograms of Crude Phosphonoacetate Uridine 7mers Synthesized with 2'-ACE Protection; Top: Before 2'-Deprotection; Bottom: After 2'-Deprotection.

In both cases, the deprotection conditions caused a large reduction in retention time, will little full length product remaining. The change in retention time was so drastic that the majority of both samples eluted at about the time flow-through material could be seen, indicating almost no binding to the column matrix. ESI-MS and MALDI-TOF analysis of the same samples produced inconclusive results as no assignable masses were found.

Therefore, in an attempt to obtain a better mass spectrum, the deprotection reaction was repeated on a purified, uridine-3'acetyl thymidine phosphonoacetate dimer protected with the ACE group at the 2' position. This compound was prepared as described in the previous chapter using compound Xa and reacting it with 3'-acetyl thymidine, oxidizing with CSO, then removing the DMCE group with DBU

followed by HPLC purification. **Figure 3.13** shows a reverse phase HPLC chromatograph before and

after treatment with 100mM acetic acid, pH 3.8 for 30 minutes at 55°, and once again a decrease in

retention time was seen.
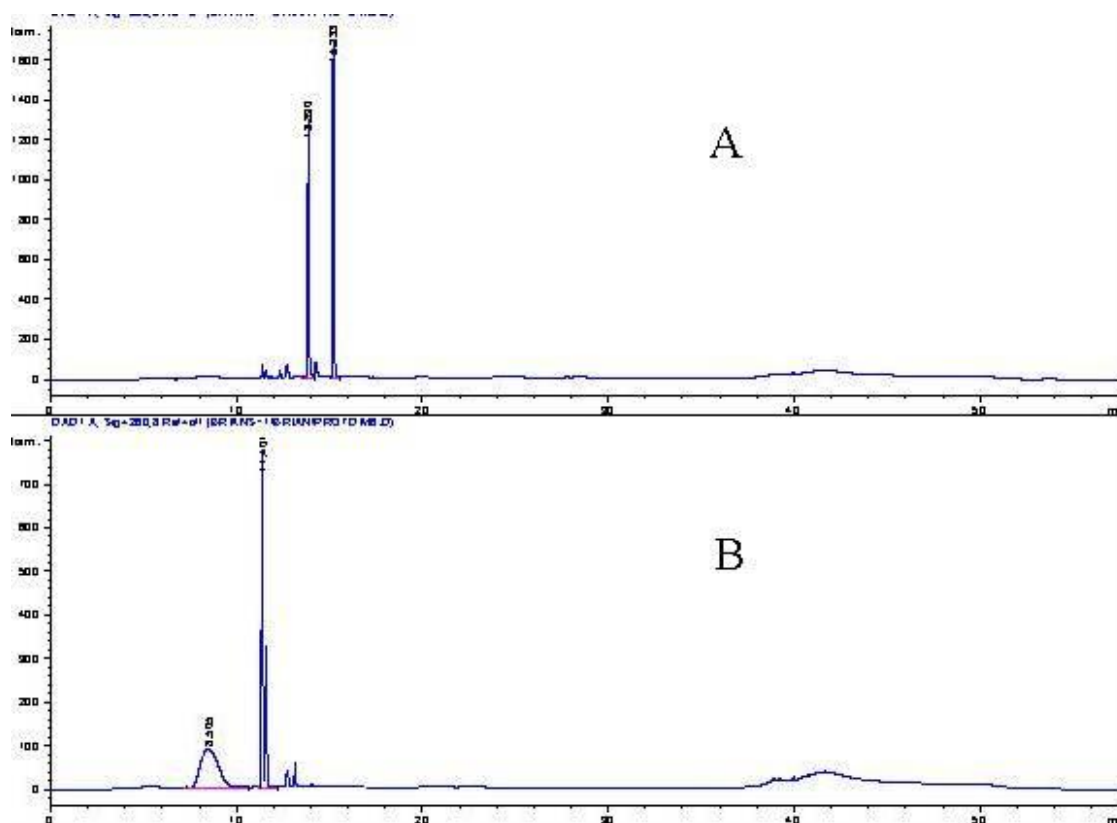
Figure 3.13: Reversed Phase HPLC Chromatograms of an HPLC Purified, 2'-ACE Protected Uridine-3'-*O*-Acetyl Thymidine Phosphonoacetate Dimer Before and After 2'-Deprotection; A: Before Deprotection; B: After Deprotection.

Additionally, the ^{31}P NMR of the crude samples showed a shift from the expected 30-28 to a peak at 19.7

ppm (**Figure 3.14**).
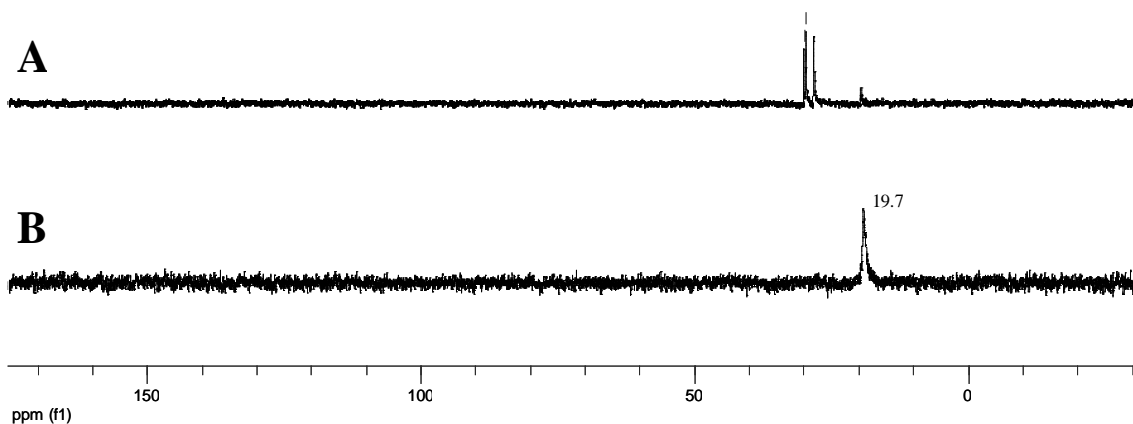
Figure 3.14: **^{31}P NMR of Crude Phosphonoacetate Uridine Thymidylate Dimer Synthesized with 2'-ACE Protection; A: Before 2'-Deprotection Showing Peaks at 30-28 ppm; B: After 2'-Deprotection Showing Peaks at 19.9-19.7 ppm.**

ESI-MS analysis of the sample showed a mass at 404.9 corresponding to 3'-*O*-Acetyl thymidine with a 5'-phosphonoacetate monoester as well as a mass of 754.18 corresponding to the ribonucleoside with a 3'-phosphonoacetate monoester (**Figure 3.15**) indicating degradation of the phosphonoacetate linkage upon 2' deprotection.
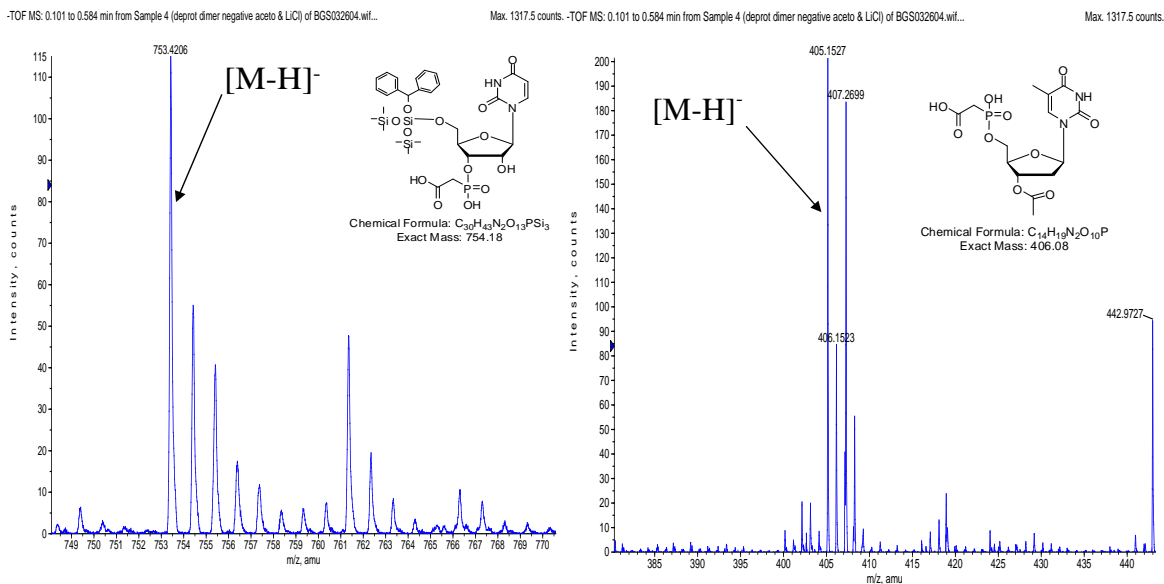
Figure 3.15: ESI-MS Spectrum of the Uridine-3'-Acetyl Thymidine Phosphonoacetate Dimer Deprotection Mixture Showing both a 5'- and 3'-Phosphonoacetate Monoester as Degradation Products.

3.3.   Discussion

Using phosphinoamidite monomers having a 5' BzH group in combination with a 2' MTHP or 2' ACE protecting group (compounds **Xa** and **Xb**), it is possible to synthesize uridinyl homopolymers containing the phosphonoacetate backbone linkage on a solid support. Interestingly, higher coupling efficiencies were seen while using the ACE protecting group as compared to the MTHP group. Despite its smaller overall size, the MTHP group may show lower coupling yields because of its local steric bulk. Specifically, when compared to the ACE group, the MTHP functionality has a tertiary carbon bearing a methoxy and two methylene groups directly connected to the 2'-hydroxyl, thereby having a higher steric

load than the ACE group near the coupling reaction site of the 3'-hydroxyl. The ACE group has a

secondary carbon with no methylene groups directly attached. This lower steric load local to the 3'-

hydroxyl is one possible explanation for the superior coupling yields seen with the ACE monomer as

shown in **Figure 3.10**. It is worth noting that the broad peaks seen in both RP and IE HPLC

chromatograms are the result of the phosphonoacetate modification imparting chirality upon the

phosphorus center. As a result, a fully modified 7mer represents 64 different oligomers.

While phosphonoacetate uridine homopolymers are stable to conditions during chain elongation,

the phosphonoacetate linkage is unstable either during 2'-deprotection or after deprotection while in close

proximity to the 2'-hydroxyl. It is possible that even the mild conditions under which the ACE group is

removed were acidic enough to cause chain cleavage via a mechanism similar to the acid catalyzed

transesterification seen with unmodified RNA oligomers (**Figure 3.16**).
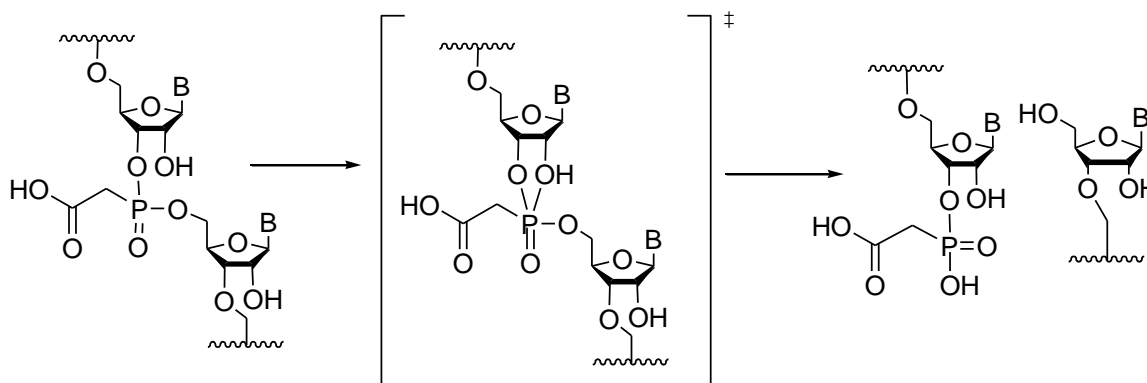
Figure 3.16: Possible Mechanism for Phosphonoacetate RNA Degradation When Treated with Acid.

In this mechanism, protonation of the phosphate backbone is the first step. The pKa of the

phosphodiester is approximately 1, whereas Sheehan et al. has measured the pKa for the

phosphonoacetate linkage as 3.4 (Sheehan, Lunstad et al. 2003). Therefore, if degradation of the

phosphonoacetate linkage is proceeding by a similar mechanism, exposing it to acidic conditions at a pH

close to its pKa would certainly be conducive to degradation.

Experiments attempting to 2'-deprotect a uridine-thymidine phosphonoacetate dimer demonstrated that the degradation products of the deprotection reaction are a 5'- and 3'-phosphonoacetate monoester (**Figure 3.7**). This product fits with the mechanism of acid catalyzed transesterification, however further mechanistic studies would be needed to confirm this hypothesis.

To address the use of both the 2'-silyl and 2'-acid labile approaches to phosphonoaceteate synthesis it is worth addressing other phosphorous-carbon bond containing linkages. Current methods for the 2'deprotection of a silyl group, either TOM or TBDMS use much milder reagaents. The most commonly used is TEA:3HF (Wincott, DiRenzo et al. 1995). Conditions such as these where the pH of the deprotection mixture can be better controlled should be further examined to look at the stability of phosphonoacetate oligomers.

Previous attempts to synthesize methylphosphante RNA also proved to be unsuccessful (Marugg, Vroom et al. 1986). Using solution phase synthesis, methylphosphonate dimers using a 2' acid labile protecting group, resulted in similar results. The methylphosphonate group was cleaved upon 2' deprotection. In addition to this, cleavage from support prior the DMCE protecting group removal on the phosphonoacetate linkage results in some degradation even in phosphonoacetate DNA. This indicates an inherent instability to nucleophillic attack upon a phosphonate linkage within a nucleic acid oligomer.

3.4.   Materials and Methods

3.4.1.   Synthesis of Phosphonoacetate Oligoribonucleotides

Solid phase synthesis of phosphonoacetate oligomers was performed on an ABI 392 automated DNA/RNA synthesizer from Applied Biosystems (Foster City, CA). The synthesizer was modified by

removal of the glass flow restrictor fittings to avoid reactions with the fluoride ion. All polystyrene support columns were purchased from Dharmacon Research (Lafayette, CO). All other DNA/RNA synthesis reagents including activators and capping reagent A were purchased from Glen Research (Sterling, VA). All solid phase syntheses were performed on a one micromole scale and the cycle was adapted from a standard one micromole phosphonoacetate DNA synthesis cycle (Scaringe, Wincott et al. 1998) with the coupling time increased to 3996 secconds (66.6 min.). Phosphinoamidite monomers (0.1M in anhydrous acetonitrile), capping solution B (0.625% w/v *N,N*-dimethylaminopyridine in anhydrous pyridine), oxidant (0.1M in anhydrous acetonitrile) and activator (0.25M ETT in anhydrous acetonitrile) were all prepared immediately prior to use. The 5'-deprotection mixture (15% TEA, 3% *N*-methyldiethanolamine, 3% H_2O, 4% hydrofluoric acid (48% aq.) in DMF) was kept for no longer than five days after preparation. Deprotection of the 5' hydroxyl was accomplished with a 35 second delivery of the fluoride solution followed by a 30 second wash with DMF.

After synthesis, polystyrene columns were washed with anhydrous acetonitrile and dried with argon. Anhydrous DBU (stored over molecular sieves) was diluted to 1.5% with anhydrous acetonitrile and applied to the column between two, 1.0 mL syringes. This was carried out for 60 minutes by pushing the solution back and forth through the column. The polystyrene was washed thoroughly with acetonitrile, dried extensively with argon and placed in a one dram vial. 40% methylamine (aq.)(1.5 mL) was added and the vial sealed with a teflon lined screw cap. The vial was then placed in a 55°C heating block and allowed to react for 1 hour. Following cleavage, the vial was cooled on ice, the polystyrene removed by filtration, and the supernatant evaporated to near dryness *in vacuo.*

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The ACE deprotection mixture was prepared by diluting glacial acetic to 100mM and adjusting the pH to 3.8 by addition of TEMED. The MTHP deprotection solution was prepared by diluting glacial acetic acid to 80% in deionized water. 2'-Hydroxyl deprotection reactions were performed in a one dram

vial by adding 1.0 mL of the deprotection mixture to the crude oligonucleotide and incubating at 55°C. After deprotection, the reaction mixtures were evaporated *in vacuo* to one half the original volume and diluted to 1.0 mL with 100mM triethylammonium acetate buffer and immediately analyzed.

3.4.2. RP and IE HPLC of Phosphonoacetate Oligomers

For reverse phase HPLC analyses, oligomers were analyzed on an Agilent 1100 series HPLC instrument using a Hypersil ODS 4.0 X 250mm C-18 column. Eluents were: (A), 100mM triethylammonium acetate in water, pH 7.5; (B), acetonitrile. Eluent gradient was 0-30% B in 32 minutes at a flow rate of 1.0 mL/min. Effluent was monitored at 260nm. Relative areas of each peak were determined by integration, following correction for changes in extinction coefficient.

For anion exchange HPLC, oligomers were analyzed on an Agilent 1100 series HPLC instrument using a Dionex DNAPac PA200 4.0 X 250mm column. Eluents were: (A), 5.0mM Tris, 5.0mM $NaClO_4$, 3.0mM $HClO_4$, 380mM CH_3CN, pH 7.5; (B), 5.0mM Tris, 344mM $NaClO_4$, 3.0mM $HClO_4$, 380mM CH_3CN, pH 7.5. The eluent gradient was 0-70% B in 42 minutes at a flow rate of 1.0 mL/min. with a column temperature of 55°C. Effluent was monitored at 260nm. Relative areas of each peak were determined by integration, following correction for changes in extinction coefficient.

3.4.3. ^{31}P NMR of Phosphonoacetate Oligoribonucleotides

All ^{31}P NMR data was obtained on a Varian 400 MHz spectrometer using an internal deuterium oxide capillary. For a 1.0μM synthesis, typical acquisition time was 80 minutes with the sample at an approximate concentration of 2μM.

CHAPTER IV

SYNTHESIS AND CHARACTERIZATION OF NUCLEOSIDES HAVING A PEROXY ANION LABILE 5'- PROTECTING GROUP

4.1. Background

As discussed in chapter 1, the successful development of a two-step approach for the solid phase synthesis of DNA has created an interest in formulating a similar strategy for RNA synthesis. Because of the simplicity and minimal financial cost of the reagents used in the two-step cycle its application to RNA synthesis was an attractive challenge. Additionally the irreversible nature of the 5'-deprotection reaction allows for this chemistry to be used in applications such as synthesis of RNA on a two dimensional surface and large scale RNA synthesis.

Development and extensive optimization of the two-step DNA synthesis chemistry resulted in the use of an aryl carbonate to mask the 5'-hydroxyl. It was removed following each round of synthesis with a mildly basic solution of peroxy anions. This peroxy anion solution also simultaneously oxidized the phosphite triester to the phosphate triester (**Figure 4.1**).
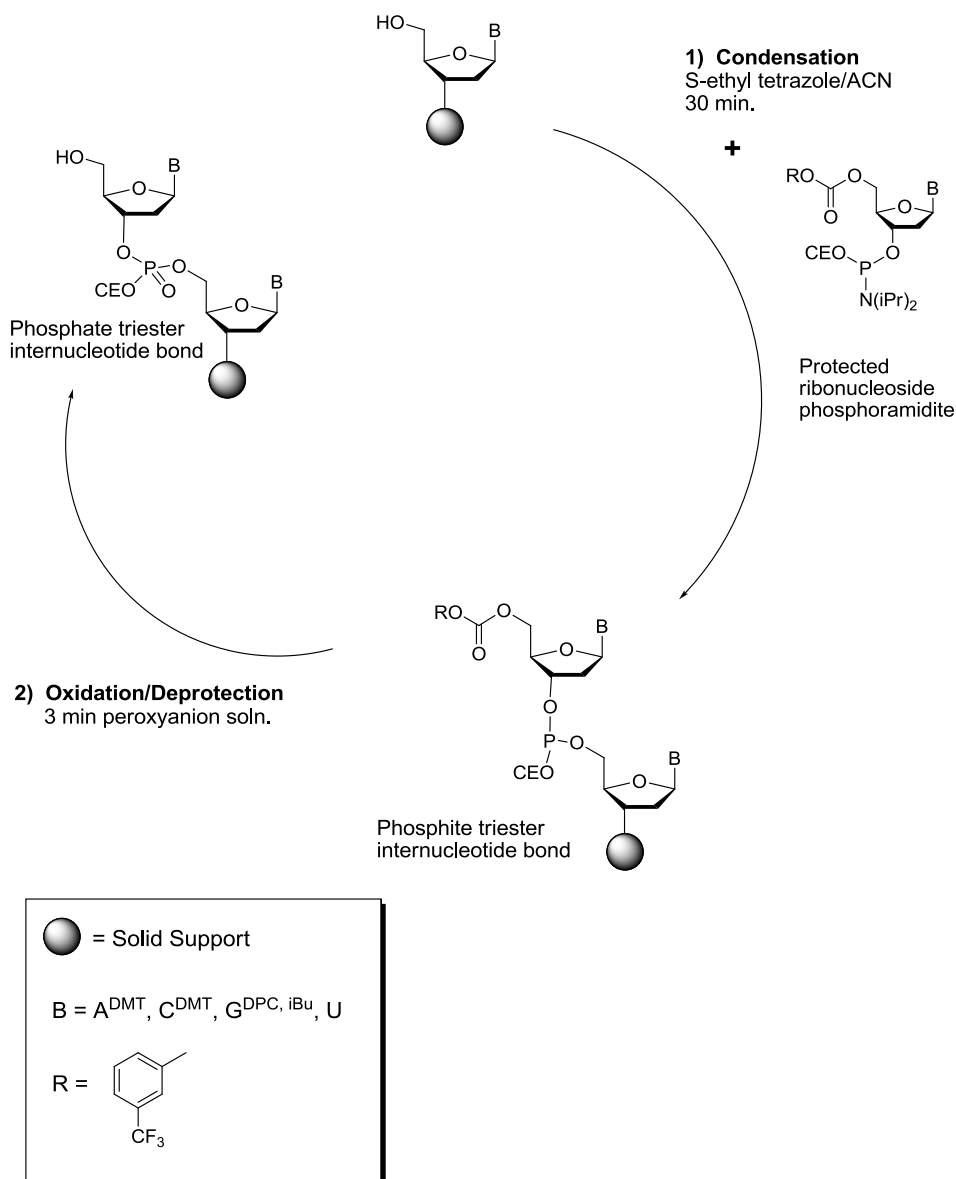
Figure 4.1: **The solid Phase Synthesis Cycle Used for the Two-Step Synthesis of Deoxyoligonucleotides.**

While designing and attempting an orthogonal protecting group scheme for RNA synthesis, several levels of complexity were encountered. As discussed in previous chapters, the oligomer was exposed to the reagent used to remove the 5' hydroxyl each time the oligomer was elongated which required that the other protecting groups on the synthons (nucleobase, phosphate and 2'-hydroxyl) have orthogonal stability. The adaptation of the two-step cycle to RNA synthesis outlined in this chapter is the

result of maintaining as much of the two-step DNA synthesis chemistry constant, while finding

appropriate protecting groups for the 2'-hydroxyl and nucleobase functionalities. The resulting approach

allowed this application-based chemistry to be used in the areas to be discussed in the following sections.

One application for the two-step RNA synthesis methodology is to synthesize RNA oligomers on

a planar glass surface for use in high density RNA arrays. These arrays would be created by spotting

RNA synthesis reagents directly onto a glass surface and the oligomer elongated. Following synthesis,

the protecting groups are removed; however, the oligomer remains bound to the glass surface and never

purified. This application limits the choice of a 2'-protecting group for the following two reasons. The

two-step synthesis strategy, as it was with DNA synthesis, utilizes an aryl carbonate as the 5'-blocking

group. Peroxy anions were used because of their increased nucleophilicity at lower than Bronsted

predicted pH values due to the alpha effect of peroxy anions (Herschlag and Jencks 1990).

Efficient removal of the 5'-carbonate required a deprotection solution having a pH of 9.6. Thus

the basicity of the peroxy anion solution required the use of a base stable blocking group at the 2'-

position. These may be labile to other reagents such as fluoride (silyl) or acid (acetal, orthoester etc.).

Silyl protecting groups are removed with fluoride ion which reacts with glass. Because the desired

approach is to synthesize the RNA oligomer on the glass support and deprotect the 2'-hydroxyl while the

oligomer is support-bound, silyl blocking groups were not an option for 2'-protection. Therefore our

focus was an acid labile 2'-protection strategy. Previous attempts using a 2'-ACE protection scheme

demonstrated that the ACE group was unstable to the peroxy anion solution used to deprotect the

arylcarbonate (Sierzchala, Dellinger et al. 2003). In light of these results, the protecting group 1,3-

benzodithiol-2-y1 (**Figure 4.2**) was tested for stability.
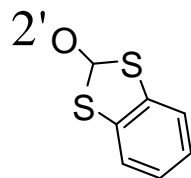
Figure 4.2: Structure of the 1,3-Benzodithiolylium (BDT) Group.

       The BDT group was reported to have a $t_{1/2}$ of 30 minutes in 80% aqueous acetic acid when tested for stability while attached to a 5'- hydroxyl (Reese and Skone 1985). If the BDT showed similar lability on the 2'-hydroxyl of a nucleoside and stability toward the peroxy anion solution, it would serve as a suitable blocking group for use during two-step synthesis.

       Another consequence of the basicity of the peroxy anion solution was that the standard, base labile nucleobase protecting groups used on adenosine and cytidine (isobutyryl on adenosine and acetyl on cytidine) could not be used as they are removed when exposed to the deprotection solution (Sierzchala, Dellinger et al. 2003). Therefore in the two-step approach to DNA synthesis, the acid labile DMT group was used to protect the exocyclic amines on both adenosine and cytidine. The DMT group was removed post-synthesis by a treatment with 3% TCA in dichloromethane for two hours. This exocyclic amine protection could not be used for RNA synthesis because such acidic treatment would deprotect the 2'-BDT group and most likely promote strand cleavage via acid catalyzed transesterification. For these reasons, 2,4,6-trimethylbenzoyl, (which was previously shown to be stable to the peroxy anion deprotection solution) was used for the exocyclic amine protection on adenosine and cytidine (Timar 2003).

       To summarize, the overall protection strategy in the two-step approach to RNA synthesis was to use an aryl carbonate to protect the 5' hyrdroxyl and remove it with peroxy anion solution following each round of synthesis. The other remaining protecting groups are stable to the peroxy anion solution: 2,4,6-trimethylbenzoyl on the exocyclic amines of cytidine and adenosine, isobutyryl on guanosine and BDT on

the 2'-hydroxyl. The nucleobase protecting groups could then be removed with ammonium hydroxide following synthesis during cleavage from support and the BDT removed with subsequent acid treatment.

An investigation of various substituted arylcarbonates for use in two-step DNA synthesis showed that 3-(trifluoromethyl)phenyl arylcarbonate possessed the highest lability to the peroxy anion solution (Sierzchala, Dellinger et al. 2003). These results prompted us to use the 3-(trifluoromethyl)phenyl arylcarbonate protecting group in two-step RNA synthesis as well. Additional arylcarbonate protecting groups were screened as potential candidates for use on the 5'-hydroxy. This was necessary because very low selectivity for the 5'-hydroxyl was observed while introducing the arylcarbonate to the nucleosides. Reaction yields below 33% were observed while reacting the 3-(trifluoromethyl)phenyl chloroformate selectively to the ribonucleoside at the primary 5'-alcohol . This observation prompted us to search for increased selectivity to the 5'-hydroxyl, we therefore screened other bulkier protecting groups for peroxy anion lability.

4.2.   Results

4.2.1.   5'-Arylcarbonate 2'-BDT Protected Nucleoside 3'-Phosphoramidites

Because of its known reactivity towards peroxy anions and successful utilization in two-step DNA synthesis, the 3-(trifluoromethyl)phenyl (FARCO) derivative was chosen as the 5'-arylcarbonate protecting group for the ribonucleoside 3'-phosphoramidite synthons (Sierzchala, Dellinger et al. 2003). The uridine 3'-phosphoramidite monomer (XV) was synthesized according to the scheme in **Figure 4.3**.
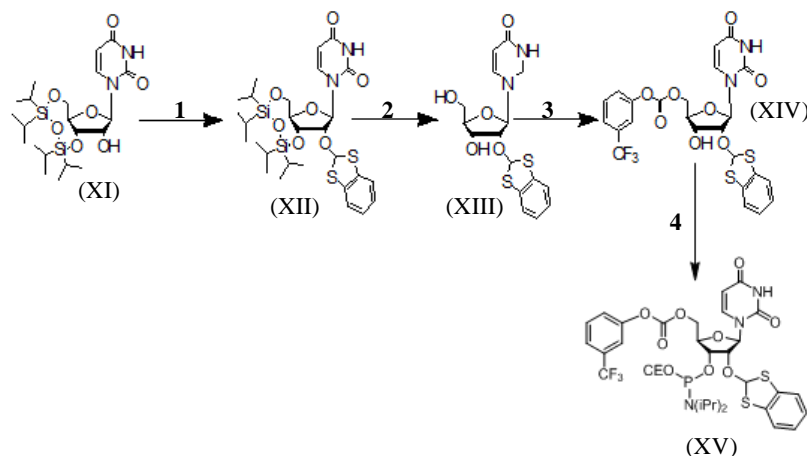
Figure 4.3: Synthetic Route Used to Prepare 5'-O-[3-(trifluoromethyl)phenoxy]carbonyl-2'-O-BDT-Uridine Phosphoramidite : 1: 1,3-Benzodithiolylium Tetrafluoroborate /Pyr; 2: TEA•3HF/Pyr; 3: 3-(Trifluoromethyl)phenyl Chloroformate/Pyr; 4: *N'N'N'N'*-Tetraisopropyl-*O*-Cyanoethyl Phosphane/DCI/DCM.

The BDT group was first introduced to the 2'-hydroxyl of 5',3'-O-(TIPS)-uridine (XI) under basic conditions using the tetrafluoroborate salt of the BDT cation. The TIPS group was removed with fluoride ion and 3-(trifluoromethyl)phenyl chloroformate was added to form the 5'-arylcarbonate. Due to poor selectivity for the 5'-hydroxyl (yields of 15-20% at 100mM, room temperature), the reaction was performed as a very dilute concentration (10 mM) at -15°C while adding the chloroformate slowly (90 minutes). Yields of 30-35% were typically obtained underthese conditions. The ribonucleoside was then phosphitylated to form the phosphoramidite (**XV**).

To synthesize the cytidine and adenosine 3'-phosphoramidites, the scheme in **Figure 4.4** was used.
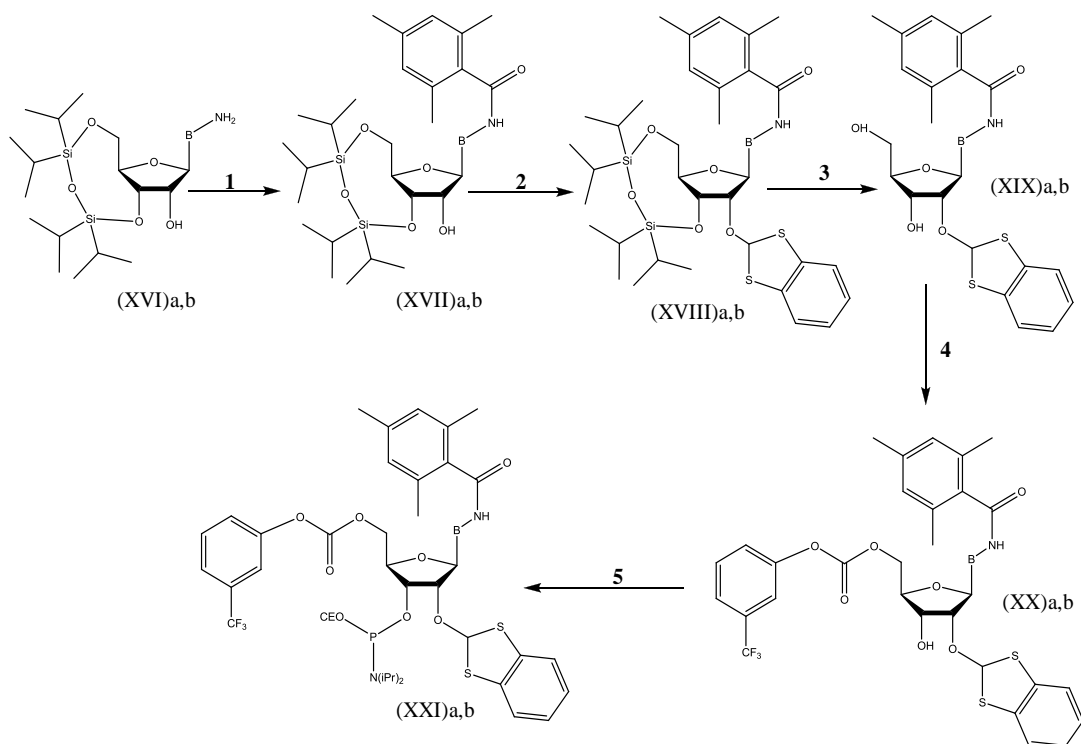
Figure 4.4: Synthetic Route Used to Synthesize 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT -*N*^{4}-2,4,6-trimethylbenzoyl cytidine (XXIb) and 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-*N*^{6}-2,4,6-trimethylbenzoyl-adenosine phosphoramidites (XXIa); 1:Chlorotrimethylsilane/Pyr/ 2,4,6-Trimethylbenzoyl Chloride/DIPEA; 2: BDT•BF4/Pyr; 3: TEA•3HF/Pyr; 4: 3-(Trifluoromethyl)phenyl Chloroformate/Pyr; 5: *N'N'N'N'*-Tetraisopropyl-*O*-cyanoethyl Phosphane/DCI/DCM. B = Adensosine(a) or Cytidine(b).

In the case of guanosine, protection of O^6 on the nucleobase was necessary due to the lack of a capping step in the solid phase synthesis cycle. This is necessary as it has been reported that during chain elongation, O^6 branching is seen when a capping step is not employed. This branching is a result of phosphoramidite coupling to the O^6 enol tautomer of guanosine. This reaction is reversed during exposure to the capping reagents (Beaucage and Iyer 1992). As a consequence, protection of the O^6 position is carried out with *N,N*-diphenylcarbamoyl group. In addition, the N^2 position is protected with an isobutyryl group. As seen in **Figure 4.5**, carbamoylation was carried out on the 5',3'-*O*-(TIPS)-N^2-iBu-guanosine.
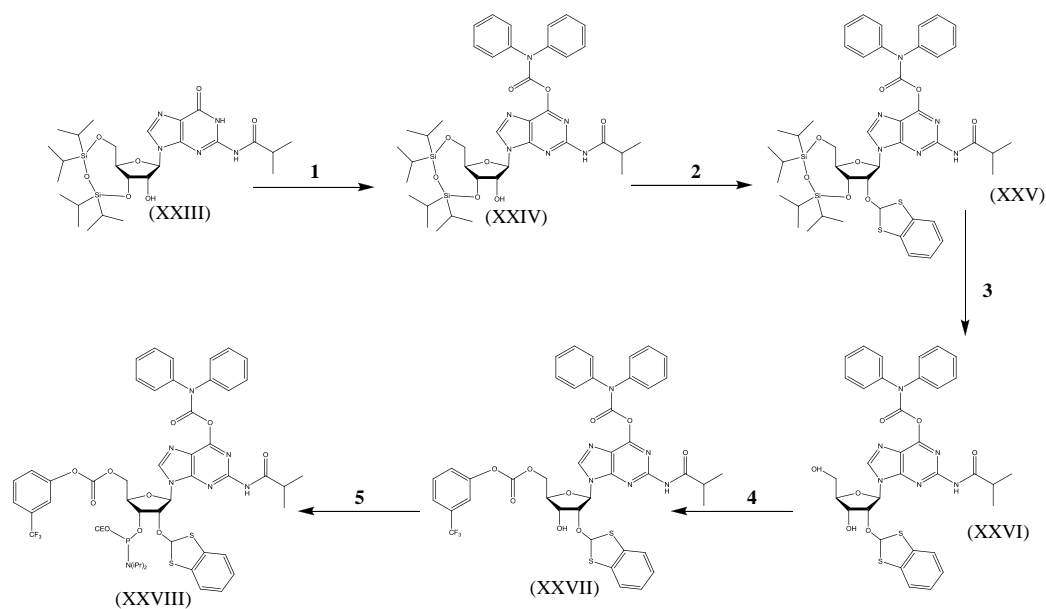
Figure 4.5: Synthetic Route Used to Synthesize 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT - *N*²-isobutyryl-*O*⁶-(N,N-diphenyl)carbamoyl guanosine phosphoramidite (XXVIII); 1: TEA/N,N-Diphenylcarbamoyl chloride; 2: 1,3-Benzodithiolylium Tetrafluoroborate /Pyr; 3: TEA•3HF/Pyr; IV: 3-(Trifluoromethyl)phenyl Chloroformate/Pyr; 4: *N'N'N'N'*-Tetraisopropyl-*O*-cyanoethyl Phosphane/DCI/DCM.

The remainder of the synthesis proceeded similarly with the other three ribonucleosides. The final step of the synthesis is to phosphitylate the 3'-hydroxyl on all four protected ribonucleosides with *N'N'N'N'*-tetraisopropyl-O-cyanoethyl phosphane using dicyanoimidazole as an activator.

The BDT group proved to be labile to the acidity of the silica matrix used during column chromatography. Therefore following introduction of the BDT group to the 2'-hydroxyl, it was necessary to neutralize the silica gel in every subsequential step with either TEMED or triethylamine. Similarly when concentrating the pyridine solution used during the 5'- carbonate formation, pyridinium hydrochloride salts appeared to be acidic enough to remove the BDT group. Improved yields were obtained when these solutions were not concentrated to complete dryness.

4.2.2.   5'- Arylthiocarbonates, 5'- Silanes and 5'- Siloxanes

Introduction of the FARCO group at the 5'- hydroxyl of ribonucleosides consistently resulted in very poor yields (30-40%) due to poor selectivity for the 5'-hydroxyl over the 3'-hydroxyl. This large reduction in yield prompted us to explore alternative peroxy anion labile protecting groups that could be introduced more efficiently at the 5'-hydroxyl. Two classes of groups were examined in order to accomplish this: 5'-arylthiocarbonates and 5'-silyl protecting groups. We hypothesized that substituted phenylchlorothiolformates would be slightly less reactive towards alcohols due to the sulfur substitution and therefore more selective for the 5'-primary alcohol. The resulting arylthiocarbonate would still be labile to peroxy anions. However, in order to test the lability of arylthiocarbonates, two model compounds were synthesized, 5'-O-S-phenylcarbonothioate-2'-O-deoxythymidine and 5'-O-[4-(Trifluoromethyl)- S-phenylcarbonothioate]-2'-deoxythymidine as outlined in **Figure 4.6**.

Large, silyl-based protecting groups were also of interest because bulky, substituted alkyl silanes and siloxanes can be introduced selectively to the 5'- hydroxyl of nucleosides (Scaringe 1996). Alkyl silane- and siloxane ethers are also susceptible to nucleophilic attack under basic conditions (Scaringe 1996). To assess the peroxy anion lability of three silyl protecting groups, three model compounds were synthesized: 5'-*O*-triphenylsilyl-2'-deoxyuridine, 5'-*O*-triethylylsilyl-2'-deoxyuridine and 5'-O-t-butoxylsiloxyl-2'-deoxyuridine as outlined in **Figure 4.6**.
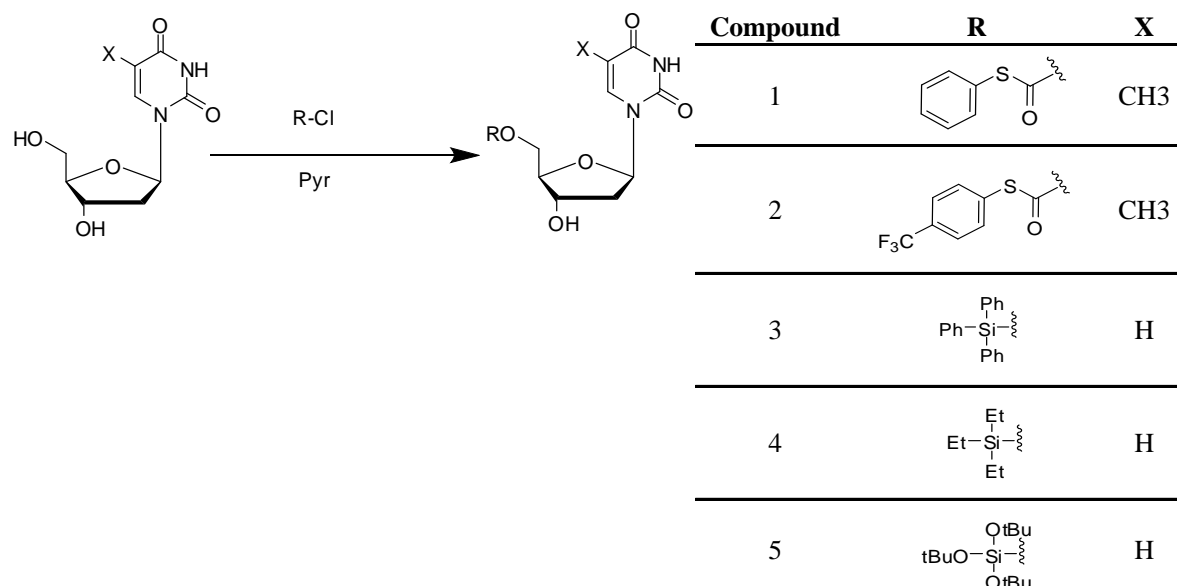
Figure 4.6: Synthesis and Structures of 5'- Protected Deoxynucleosides Synthesized to be Assayed for Lability to the Peroxy Anion Solution.

4.2.3. Peroxy anion Lability Studies

To assess the lability of various protecting groups, each of the nucleosides 1-5 was treated with the peroxy anion solution and TLC analysis was performed at various time points in order to judge when deprotection was complete. For comparison, a 5'-FARCO 2'-deoxythymidine nucleoside was also analyzed. Only protecting groups showing lability comparable to the FARCO group were considered for use in solid phase synthesis. Results of the deprotection assay are shown in **Table 4.1**.

Compound	5' Protecting group removal Time
1	>3 min.
2	1.5 min.
3	<1 min.
4	<1 min.
5	> 30 min.

Table 4.1: **Reaction Times for Complete Removal of the 5'- Protecting Groups with the Peroxy Anion Solution.**

The FARCO group was removed in under one minute. The only other protecting groups showing

similar lability under these conditions were the triethyl- and triphenylsilyl ethers, however they both

showed equivalent or less selectivity to the 5' hydroxyl and instability during attempts to

chromatographically separate the isomers. The FARCO group was chosen for solid phase RNA synthesis

studies due to its proven effectiveness in solid phase DNA synthesis.

4.3.   Discussion

The syntheses of four protected 5'-FARCO- 3'-phosphoramidites for use in two-step RNA

synthesis were successfully accomplished. During the synthesis of these monomers, it was seen that the

BDT group used to protect the 2'-hydroxyl was observed to be very labile during silica column

chromatography at all stages of the synthesis. For this reason, great care was taken to keep the silica gel

basic using triethylamine or TEMED. The BDT group was also observed to be partially removed in the

presence of pyridinium hydrochloride. Therefore, reactions resulting with the formation of this salt were

never concentrated to dryness. This salt was removed with a bicarbonate wash. The introduction of the

5'-carbonate proved to be the most difficult transformation. Even when the reaction was performed while

extremely dilute (10 mM) at -15°C, poor selectivity (35%) for the 5'- hydroxyl was observed. As a result,

the desired 5'-FARCO ribonucleosides required a difficult chromatographic separation from both the 3'-FARCO product as well as the bis-5',3'-FARCO product.

An attempt to find other peroxy ion labile protecting groups showed promise, but superior selectivity to the 5'-hydroxyl over the 3'-hydroxyl was not achieved. Stability during chromatographic purification also presented a problem. The end result was to choose the proven FARCO group for 5' hydroxyl masking during solid phase synthesis.

4.4.   Materials and Methods

4.4.1.   General Procedures

Procedure A. 2'-*O* -Protection of nucleosides with 1,3-benzodithiolylium (BDT) group:

In a 500 mL round-bottomed flask, nucleoside (10 mmol), pyridine (30.0 mmol) and 1,3-benzodithiolylium tetrafluoroborate (15.00 mmol) were combined in dichloromethane (350 ml). The flask was covered in aluminum foil and the reaction allowed carried out at room temperature for 48 hours. Triethylamine was added and the reaction continued for an additional hour. The reaction mixture was concentrated *in vacuo*, co-evaporated twice with toluene, resuspended in dichloromethane (100 mL) and washed with 150mM sodium phosphate (pH 7.5) and saturated NaCl. The organic layer was dried over sodium sulfate, concentrated and loaded onto a silica column.

Procedure B. Removal of 5,'3'-*O-N,N,N,N*-tetraisopropyldisiloxoyl (TIPS) group:

The nucleoside (10 mmol) was dissolved in minimal acetonitrile (approximately 100 mL)and pyridine (1750 mmol) was added. Triethylamine trihydrofluoride (35.0 mmol) was slowly injected into the reaction vessel by syringe. The reaction was monitored by TLC (4% MeOH/DCM) and allowed to continue until all starting material was consumed (typically around 3 hours). At this time the reaction

mixture was concentrated to near dryness *in vacuo*, resuspended with dichloromethane and added to a silica gel column. The product was eluted with a gradient of 1-8% methanol/dichloromethane/0.1% triethylamine.

Procedure C. 5'-*O*-Protection of nucleosides with [3-(trifluoromethyl)phenoxy]carbonyl (FARCO):

In a jacketed 1 L round-bottomed flask, nucleoside (10 mmol) was dissolved in pyridine (800 ml) and cooled to -15 °C using an ethylene glycol chiller. In a dropping funnel, 3-(trifluoromethyl)phenyl chloroformate (10 mmol) was dissolved in dichloromethane (100mL) and added dropwise to the nucleoside over 90 minutes. The reaction was allowed to proceed overnight at -15 °C. At this time it was brought to room temperature and concentrated in vacuo. The reaction mixture was diluted with dichloromethane (200 ml) and washed with saturated sodium bicarbonate followed by saturated sodium chloride. The organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo*. The product was purified via silica gel chromatography.

Procedure D. Phosphitylation of nucleosides:

Protected nucleoside (10 mmol) was dissolved in 200mL of dichloromethane *N'N'N'N'*-Tetraisopropyl-*O*-cyanoethyl phosphane (12.00 mmol) and DCI (11.00 mmol) were added and the reaction mixture stirred at room temperature for 10-12h. The reaction was neutralized with *N,N,N,N*-tetramethylethylenediamine (11 mmol), concentrated in vacuo, and the crude reaction mixture loaded directly onto a silica column without further workup.

Procedure E. N-Protection of nucleosides with 2,4,6-trimethylbenzoyl via the Jones procedure:

The nucleoside (10 mmol) was co-evaporated 3 times with anyhydrous pyridine and placed under high vacuum overnight. It was then dissolved in pyridine (300 ml). chlorotrimethylsilane (30.0 mmol) was added via syringe and allowed to react for 30 minutes at room temperature. Diisopropylethylamine

(20 mmol) and 2,4,6-trimethylbenzoyl chloride (20 mmol) were added and allowed to react for 18 hours. Water (80 ml) was added and the reaction mixture stirred at room temperature for 3 hours. The water/pyridine solution was concentrated *in vacuo*, co-evaporated with toluene, redissolved in ethyl acetate, and washed with saturated sodium bicarbonate and brine. The organic layer was dried over sodium sulfate, filtered, concentrated *in vacuo,* purified by silica column chromatography.

4.4.2.   Synthesis of nucleosides

Preparation of 5',3'-*O*-(TIPS)-2'-*O*-BDT-Uridine:

5',3'-*O*-(TIPS)- uridine (4.87 g, 10 mmol) was 2'-*O*-protected according to procedure A. The crude reaction mixture was added to a silica gel column and the product eluted with a gradient of 10-40% ethyl acetate/hexanes/0.1% TEA. Pure product (5.88 g, 92% yield) was analyzed by ^{1}H NMR and ESI mass spectrometry. ^{1}H NMR (CDCl3) 7.60-7.21(m 4H), 7.19(d 1H j=8.1), 7.06(s 1H), 6.02(d 1H j=4.8), 5.72(d 1H j=8.1), 5.42(d 1H j=5.4), 5.12(d 1H), 4.13(m 1H), 4.01(m 1H), 3.91-3.67(m 3H) 1.3-0.99(m 28H). ESI MS 639.2 [M+H]+.

Preparation of 2'-O-BDT-Uridine:

5',3'-*O*-(TIPS)-2'-*O*-BDT-uridine (6.39g, 10 mmol) was deprotected according to procedure (B). The crude reaction mixture was added to a silica gel column and the product eluted with a gradient of 1-12% methanol/dichloromethane/0.1% triethylamine. Pure product (3.33g, 86% yield) was characterized by 1H NMR and mass spectrometry. [1]H (DMSO) 11.38(s br 1H), 7.5-7.15(m 4H), 7.75(d 1H j=8.1), 7.04(s 1H), 5.87(d 1H j=4.8), 5.64(d 1H j=8.1), 5.32(d 1H j=5.3), 5.01(d 1H), 4.14(m 1H), 3.97(m1H), 3.82(m 1H), 3.51-3.47(m 2H). ESI MS 383.1 [M+H]+

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-Uridine:

2'-*O*-BDT-uridine (3.82g, 10 mmol) was 5'-*O* protected according to procedure (C). The reaction mixture was added to a silica gel column and the product eluted with a gradient of 5-40% ethyl acetate/hexanes/0.1% pyridine. Pure product (1.99g, 34% yield) was characterized using 1H NMR and mass spectrometry. ^{1}H NMR (DMSO) 9.80 (s 1H), 7.58(m 3H), 7.45(m 1H), 7.34 d 1H, j=8.1), 7.30(m 2H), 7.11(m 2H), 6.70(s 1H), 5.75(d 1H j=8.1), 5.71(d 1H j=7.32), 5.39(t 1H j=5.31), 4.74(m 1H), 4.36(m 1H), 3.87-3.76(m 2H), 4.14(m 1H). ESI MS: 591.2 [M+H]+.

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-3'-*O*-[(2-cyanoethyl)-N,N-diisopropylaminophosphino] Uridine: 5'-*O*-[3-(Trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-uridine (1.0g, 1.71mmol) was 3'-*O* phosphitylated according to procedure D. The crude reaction mixture was added to a silica gel column and the product eluted with a gradient of 0-50% ethyl acetate/hexanes/0.1% TEMED. Pure product (1.20g, 89% yield) was characterized using ^{31}P NMR and mass spectrometry. ^{31}P NMR (CDCl3): δ 150.5 & 149.3. ESI MS 791.2 [M+Li]+.

Preparation of 5',3'-*O*-(TIPS)-*N*[6]-2,4,6-trimethylbenzoyl Adenosine:

5',3'-*O*-(TIPS)- adenosine (5.1g, 10 mmol) was *N*[6]-protected according to procedure (E) The crude reaction mixture was added to a silica gel column and eluted with a gradient of 15-50% /ethyl acetate/hexanes. Pure product (5.8 g, 88 % yield) was analyzed by ^{1}H NMR and ESI mass spectrometry. ^{1}H NMR (CDCl3): 8.29(s 1H), 8.14(s 1H), 7.13(s 2H), 6.65(d 1H j=6.3), 5.32(m 1H), 3.78-3.65(3H), 2.35(s 6H), 2.21(s 3H), 1.31-1.03(m 28H). ESI MS 656.3 [M+H]+.

Preparation of 5',3'-*O*-(TIPS)-2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl Adenosine:

5',3'-*O*-(TIPS)-*N*[6]-2,4,6-trimethylbenzoyl adenosine (6.56 g, 10 mmol) was 2'-*O*-protected according to procedure (A) The crude reaction mixture was added to a silica gel column and eluted with a gradient of 10-40% ethyl acetate/hexanes/0.1% TEA. Pure product (5.74 g, 71% yield) was analyzed by ^{1}H NMR and ESI mass spectrometry. ^{1}H NMR (CDCl3): 9.54(s br 1H), 8.35(s 1H), 8.31(s 1H), 7.45-7.36(m 4H), 7.02(s 2H), 6.84(s 1H), 6.56(d 1H j=6.4), 5.02(m 1H), 4.11(m 1H), 3.67-3.61(2H), 2.24(s 6H), 2.14(s

3H), 1.21-0.99(m 28H). ESI MS 808.2 [M+H]+.

Preparation of 2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl Adenosine: 5',3'-*O*-(TIPS)-2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl adenosine (8.24g, 10 mmol) was deprotected according to procedure (B). The crude reaction mixture was added to a silica gel column and the product was eluted with a gradient of 1-10% methanol/dichloromethane/0.1% triethylamine. Pure product (4.13g, 73% yield) was characterized using 1H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 8.41(s 1H), 8.39(s 1H), 7.36(s 2H), 7.32-7.23(m 4H), 7.03(s 1H), 6.69(d 1H j=6.4), 5.19(m 1H), 3.98-3.58(m 3H), 2.32(s 9H). ESI MS 588.14 [M+Na]+.

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl Adenosine: 2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl adenosine (5.66g, 10 mmol) was 5'-*O* protected according to procedure (C). The crude reaction mixture was added to a silica gel column and the product was eluted with a gradient of 5-40% ethyl acetate/hexanes/0.1% pyridine. Pure product (2.40g, 32% yield) was characterized using 1H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 8.48(s 1H), 8.31(s 1H), 7.59-7.25(m 8H), 7.22(s 2H), 6.79(s 1H), 6.69(d 1H j=6.6), 4.45(m 1H), 4.33(m 1H), 4.26-3.56(m 3H), 2.31(s 3H), 2.26(s 6H). ESI MS 776.3 [M+Na]+.

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphino] adenosine: 5'-*O*-[3-(Trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT-*N*[6]-2,4,6-trimethylbenzoyl adenosine (1.0g, 1.32 mmol) was 3'-O phosphitylated according to procedure (D). The crude reaction mixture was added to a silica gel column and the product was eluted with a gradient of 0-50% ethyl acetate/hexanes/0.1% TEMED. Pure product (1.11g, 88% yield) was characterized using ^{31}P NMR and mass spectrometry. ^{31}P NMR (CDCl3): δ 149.9 & 148.2. ESI MS 960.5 [M+Li]+.

Preparation of 5',3'-O-(TIPS)-N4-2,4,6-trimethylbenzoyl Cytidine:

5',3'-*O*-(TIPS)- cytidine (5.0 g, 10.3 mmol) was *N*-protected according to procedure (E) The crude

reaction mixture was added to a silica gel column and eluted with a gradient of 20-60%

hexanes/ethylacetate. Pure product (5.2 g, 80 % yield) was analyzed by ^{1}H NMR and ESI mass

spectrometry. ^{1}H NMR (CDCl3): 8.22 (d 1H j=7.5), 7.66(t 1H j=7.50), 5.79(d 1H), 6.85(s 2H), 4.01(m

1H), 4.34-4.18(m 4H), 2.24(s 3H), 2.28(s 6H), 1.10-0.97(m 28H) ESI MS 632.3 [M+H]+

Preparation of 5',3'-*O*-(TIPS)-2'-*O*-BDT -*N*[4]-2,4,6-trimethylbenzoyl Cytidine:

5',3'-*O*-(TIPS)-*N*[4]-2,4,6-trimethylbenzoyl cytidine (6.32 g, 10 mmol) was 2'-*O*-protected according to

procedure (A) The crude reaction mixture was added to a silica gel column and product eluted with a

gradient of 0-40% ethyl acetate/hexanes/0.1% TEA. Pure product (6.4g, 82% yield) was analyzed by [1]H

NMR and ESI mass spectrometry. ^{1}H NMR (CDCl3): 8.65(d 1H j=7.5), 7.69(m 2H) 7.59(t 1H j=7.50),

7.34-7.45(m 4H), 6.92(s 1H), 5.92(d 1H), 4.66(m 2H), 4.64-4.59(m 3H), 2.01-1.92(m 9H), 1.20-1.01(m

28H). ESI MS 784.3[M+H]+

Preparation of 2'-*O*-BDT-N4-2,4,6-trimethylbenzoyl Cytidine: 5',3'-*O*-(TIPS)-2'-*O*-BDT -

N4-2,4,6-trimethylbenzoyl cytidine (3.92g, 5 mmol) was deprotected according to procedure (B). The

crude reaction mixture was added to a silica gel column and product eluted with a gradient of 1-10%

methanol/dichloromethane/0.1% triethylamine. Pure product (1.95g, 72% yield) was characterized using

^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 8.65(d 1H j=7.5), 7.69(m 2H) 7.59(t 1H j=7.50),

7.42-7.61(m 4H), 6.82(s 1H), 6.26(d 1H), 4.71(m 1H), 4.52-4.45(m 2H), 4.24(m 1H), 4.19(m 1H), 2.11-

1.99(m 9H). ESI MS 566.2 [M+Na]+

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT -*N*[4]-2,4,6-

trimethylbenzoyl Cytidine: 2'-*O*-BDT-N4-2,4,6-trimethylbenzoyl cytidine (5.42g, 10 mmol) was 5'-*O*

protected according to procedure (C). The crude reaction mixture was added to a silica gel column and

product was eluted with a gradient of 5-40% ethyl acetate/hexanes/0.1% pyridine. Pure product (2.04g,

28% yield) was characterized using ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 8.49(d 1H

j=7.61), 7.26(m 2H) 7.42-7.34(m 8H), 7.23(t 1H j=7.6), 7.09(s 1H), 6.13(d 1H), 4.75(m 2H), 4.57-4.44(m

2H), 1.89-1.77(m 9H). ESI MS 730.1[M+H]+

Preparation of 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT -*N*[4]-2,4,6-trimethylbenzoyl-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphino] Cytidine: 5'-*O*-[3-(trifluoromethyl)phenoxy]carbonyl-2'-*O*-BDT -*N*[4]-2,4,6-trimethylbenzoyl cytidine (1.0g, 1.7mmol) was 3'-*O* phosphitylated according to procedure (D). The crude reaction mixture was added to a silica gel column and the product was eluted with a gradient of 0-50% ethyl acetate/hexanes/0.1% TEMED. Pure product (1.0g, 78% yield) was characterized using 31P NMR and mass spectrometry. 31P NMR (CDCl3): δ 150.4 & 149.0 ppm. ESI MS 936.5 [M+Li]+.

Preparation of 5',3'-*O*-(TIPS)-*N*[2]-isobutyryl-*O*[6]-(*N*,*N*-diphenyl)carbamoyl Guanosine: In a 1 L round bottom flask, 5',3'-*O*-(TIPS)- *N*[2]-isobutyryl-guanosine (5.0 g, 8.39 mmol was dissolved in anhydrous pyridine (400 ml) and triethylamine (7.08 ml, 50.3 mmol). It was allowed to react for 15 minutes at which time *N'*,*N'*-diphenylcarbamoyl chloride was added and allowed to react for 4 hours. The reaction was quenched with water (10 ml), concentrated *in vacuo*, dissolved in dichloromethane (200 ml), and washed with sodium bicarbonate and brine. The organic layer was dried over sodium sulfate, concentrated to an oil and loaded directly onto a silica gel column. Product (5.9 g, 89 % yield) was eluted with a gradient of 5-10% ethyl acetate/hexanes. Pure product was characterized with ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 7.14(s 1H), 7.12-7.00(m 10H), 5.77(d 1H j=6.0), 4.59-4.55(m 2H), 4.43(m 1H), 3.60(m 2H), 2.01(m 1H), 1.23-1.20(m 6H). ESI MS 791.2 [M+H]⁺

Preparation of 5',3'-*O*-(TIPS)-2'-*O*-BDT - *N*[2]-isobutyryl-*O*[6]-(*N*,*N*-diphenyl)carbamoyl Guanosine: In a 500 mL round-bottomed flask, 5',3'-*O*-(TIPS)-*N*[2]-isobutyryl-*O*[6]-(*N*,*N*-diphenyl)carbamoyl Guanosine (6.5 g, 8.22 mmol) and protected according to procedure (A). Product was purified using a gradient of 0-40% ethyl acetate/hexanes/0.1% TEA. Pure product (6.90g, 89% yield) was characterized using ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 7.59-7.33(m 6H), 7.13(s

1H), 7.01-7.04(m 9H), 5.92(d 1H j=6.1), 4.52-4.55(m 2H), 4.45(m 1H), 3.41(m 2H), 2.25(m 1H), 1.27-0.89(m 36H). ESI MS: 943.3 [M+H]$^+$

Preparation of 2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl Guanosine: 5',3'-O-(TIPS)-2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl guanosine (2.0 g, 2.1 mmol) was deprotected according to procedure (B). The crude reaction mixture was added to a silica gel column and the product was eluted with a gradient of 1-8% methanol/dichloromethane/0.1% triethylamine. Pure product (1.2g, 81% yield) was characterized using ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3): 7.41-7.32(m 5H), 7.24(s 1H), 7.22(s 1H), 7.11-7.08(m 9H), 5.86(d 1H j=6.1), 4.31(m 1H), 4.52-4.46(m 2H), 3.52(m 2H), 2.54(m 1H), 1.27-1.24(m 6H) ESI MS: 701.2 [M+H]$^+$

Preparation of 5'-O-[3-(trifluoromethyl)phenoxy]carbonyl-2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl Guanosine: In a jacketed 1 L round-bottom flask, 2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl Guanosine (1.2 g, 1.72 mmol) was 5'-O protected according to procedure (C). The crude reaction mixture was added to a silica gel column and was eluted with a gradient of 10-40% ethyl acetate/hexanes/0.1% pyridine. Pure product was isolated (0.4 g, 26.3 % yield). ^{1}H NMR (CDCl3): 7.62-7.40(m 9H), 7.32(s 1H), 7.23(s 1H), 7.19-7.07(m 9H), 5.99(d 1H j=6.1), 4.29(m 1H), 4.55-4.46(m 4H), 2.66(m 1H), 1.21-1.11(m 6H) ESI MS: 607.0 [M+Na]$^+$.

Preparation 5'-O-[3-(trifluoromethyl)phenoxy]carbonyl-2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl-3'-O-[(2-cyanoethyl)-N,N-diisopropylaminophosphino] Guanosine: 5'-O-[3-(Trifluoromethyl)phenoxy]carbonyl-2'-O-BDT - N^2-isobutyryl-O^6-(N,N-diphenyl)carbamoyl guanosine (1.0g, 1.1 mmol) was 3'-O phosphitylated according to procedure (D). The crude reaction mixture was added to a silica gel column and the product eluted with a gradient of 0-50% ethyl acetate/hexanes/0.1% TEMED. Pure product (0.93g, 76% yield) was characterized using ^{31}P NMR and mass spectrometry. ^{31}P NMR (CDCl3): δ 149.8 & 148.4. ESI MS 1095.6 [M+Li]$^+$.

Preparation of 5'-*O*-*S*-phenylcarbonothioate-2'-*O*-deoxythymidine:

2'-deoxythymidine (2.42 g, 10.00 mmol) was co-evaporated 3 times with anhydrous pyridine and dried under vacuum for three hours. The 2'-deoxynucleoside was dissolved in pyridine (300 ml) and cooled to 0 °C in an ice bath. Phenylchlorothiolformate (1.72 g, 10 mmol) was dissolved in dichloromethane (50 ml), added dropwise to the 2'- deoxynucleoside and allowed to stir for four hours. The reaction mixture was concentrated *in vacuo* and twice coevporated with toluene. The resulting solid was dissolved in 200 ml of dichloromethane and washed with sodium bicarbonate and brine. The organic layer was collected and dried over sodium sulfate, concentrated *in vacuo*. The crude material was loaded onto a silica gel column and eluted with a gradient of 10-30% ethyl acetate in hexanes. Pure product (3.4 g, 90 %) was characterized by ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3) 7.42-7.31(m 5H) 7.13(s 1H), 6.11(t 1H j=6.9), 4.21-4.33(m 3H), 3.91-3.84(m 2H), 2.19(m 2H) 1.12(s 3H). ESI MS 379.2 $[M+H]^+$.

Preparation 5'-*O*-[4-(trifluoromethyl)- *S*-phenylcarbonothioate]- 2'-deoxythymidine:

In a 1.0 L round bottom flask, 2.0M phosgene in toluene (25.00 ml, 50 mmol) was diluted in anhydrous toluene (300 ml) and cooled to 0 °C in an ice bath. 4-(trifluoromethyl)thiophenol (0.98g, 10 mmol) and diisopropylethylamine (1.29g, 10 mmol) were dissolved in toluene (80 ml) and added dropwise to the phosgene solution. The reaction mixture was allowed to react for one hour and then brought to room temperature. The toluene and excess phosgene were removed *in vacuo* and the volume reduced to approximately 50 ml. This solution was diluted with anhydrous diethyl ether (100 ml) and the ammonium hydrochloride salts were removed via schlenk filtration under argon. This crude reaction mixture was used immediately without further purification or characterization. 2'-*O*-Deoxythymidine (2.42g, 10 mmol) was dissolved in pyridine (300 ml) and cooled to 0 °C in an ice bath. The S-[4-(trifluoromethyl)phenyl]chlorothiolformate was dissolved in dichloromethane (50 ml) and added to the nucleoside dropwise. This mixture was allowed to stir for four hours. At this time, it was concentrated *in vacuo* and twice co-evaporated with toluene. The resulting solid was dissolved in 200 ml of

dichloromethane and washed with sodium bicarbonate and brine. The organic layer was collected and

dried over sodium sulfate and concentrated *in vacuo*. The crude material was loaded onto a silica gel

column and eluted with a gradient of 10-30% ethyl acetate in hexanes. Pure product (3.39 g, 76 %) was

characterized by ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3) 7.84(m 4H), 7.59(t 1H j = 7.2),

6.16(t 1H j=6.7), 5.68(d 1H), 4.51(m 1H), 4.41(m 1H), 4.23(m 1H), 3.97(m 1H) 2.11(m 2H), 1.23(s 3H).

ESI MS 453.1[M+Li]$^+$.

Preparation of 5'-*O*-triphenylsilyl-2'-deoxyuridine: In a 500 ml round bottom flask, 2'-

deoxyuridine (2.28 g, 10 mmol) was dissolved in anhydrous acetonitrile (300 ml). Imidazole (1.36 ml, 10

mmol), diisopropylamine (1.42 ml, 10.00 mmol) and triphenylchlorosilane (2.95 g, 10 mmol) were added

sequentially. The reaction was monitored by TLC (4% MeOH/DCM). Upon consumption of the starting

material, the reaction mixture was quenched with water (10 ml). Acetonitrile was removed in vacuo. The

crude product was redissolved in ethyl acetate and washed with sodium bicarbonate followed by brine.

The organic layer was dried with sodium sulfate, concentrated *in vacuo*, and loaded directly onto a silica

gel column. Product was eluted with a gradient of 20-50% ethyl acetate/hexanes. The desired product

was collected as a clear oil (4.1 g, 84 % yield) and characterized by ^{1}H NMR and mass spectrometry. [1]H

NMR (CDCl3) 7.72(d 1H j = 8.0), 7.11-7.02(m 15H), 6.41(t 1H j= 6.0), 5.49(d 1H j= 8.0), 4.29(m 2H),

4.14-4.03(m 2H), 2.31(m 2H). ESI MS 488.3 [M+Li]$^+$.

Preparation of 5'-*O*-triethylylsilyl-2'-deoxyuridine:

In a 500 ml round bottom flask, 2'-deoxyuridine (2.28 g, 10 mmol) was dissolved in anhydrous

acetonitrile (300 ml). Imidazole (1.362 ml, 10 mmol), diisopropylamine (1.425 ml, 10 mmol) and

triphenylchlorosilane (2.95 g, 10 mmol) were added sequentially. The reaction was monitored by TLC

(4% MeOH/DCM). Upon consumption of the starting material, the reaction was quenched with water (10

ml) and the acetonitrile removed *in vacuo*. The material was redissolved in ethyl acetate and washed with

sodium bicarbonate followed by brine. The organic layer was dried with sodium sulfate, concentrated *in*

vacuo, and loaded directly onto a silica gel column. Product was eluted with a gradient of 20-50% ethyl

acetate/hexanes. The desired product was collected as a clear oil (4.1 g, 84 % yield) and characterized by

^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3) δ 10.01(s, br 1H), 8.02(d 1H j = 8.03), 6.39(t 1H),

5.71(d 1H j= 8.03), 4.48(m 1H), 4.07(m 1H), 3.90(m 2H), 3.82(m 1H), 2.45(m 1H), 2.16m 1H), 0.96(m

9H), 0.63(m 6H). ESI MS 365.15 [M+Na]$^+$.

Preparation of 5'-*O*-*t*-butoxylsiloxyl-2'-deoxyuridine:

In a 500 ml round bottom flask, 2'-deoxyuridine (2.28 g, 10 mmol) was dissolved in anhydrous

acetonitrile (300 ml). Imidazole (1.362 ml, 10 mmol), diisopropylamine (1.425 ml, 10 mmol) and tri-t-

butoxychlorosilane (2.83 g, 10 mmol) were added sequentially. The reaction was monitored by TLC (4%

MeOH/DCM). Upon consumption of the starting material, the reaction was quenched with water (10 ml)

and the acetonitrile removed *in vacuo*. The crude product was redissolved in ethyl acetate and washed

with sodium bicarbonate followed by brine. The organic layer was dried with sodium sulfate,

concentrated *in vacuo* and loaded directly onto a silica gel column. Product was eluted with a gradient of

10-40% ethyl acetate/hexanes. Pure product was collected as a white crystalline solid (3.1 g, 65.3 %

yield) and characterized by ^{1}H NMR and mass spectrometry. ^{1}H NMR (CDCl3) 7.80(d 1H j = 8.2), 6.32(t

1H), 5.72(d 1H j= 8.2), 4.52(m 1H), 4.02(m 1H), 2.45(m 1H), 2.23(m 1H), 1.34(s br 27H). ESI MS 481.2

[M+Li]$^+$.

4.4.3.   General Procedure for Peroxy anion Lability Studies

The nucleoside (0.01 mmol) to be tested was dissolved in 6.0 mL of the deprotection solution

and spotted onto a TLC plate at the desired time points. The TLC was eluted with 4% MeOH/DCM to

analyze the products. The peroxy anion deprotection solution was 3% (w/v) aqueous LiOH (10 mL),

1.5M 2-amino-2-methyl-1-propanol in water (15 mL), *m*-chloroperbenzoic acid (1.78 g), aqueous 30%

H_2O_2 (10 mL), and dioxane (50 mL), pH 9.6. This solution was used immediately after preparation and never kept for over 24 hours.

CHAPTER V

TWO-STEP SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES

5.1.   Background

With the advent of a successful methodology for using peroxy anions during the 5'-deprotection step of solid phase DNA synthesis, its application to solid phase RNA synthesis was explored. The lack of protic acid or fluoride ion in the synthesis cycle made two-step RNA synthesis an attractive method for use in large-scale RNA synthesis. To date, the best methodology for use in large-scale RNA synthesis is 2'-TBDMS chemistry (Muller, Wolf et al. 2004). However, even with TBDMS on the 2'-hydroxyl,large amounts of protic acid was required during each round of synthesis to deprotect the 5'-DMT group which can lead to acid-promoted depurination (Beaucage and Iyer 1992). The use of TBDMS chemistry also typically results in poor coupling yields when compared to yields seen during DNA synthesis. Developing a method to synthesize RNA oligomers using reagents compatible with "printing" high density RNA arrays on a planar surface was also of interest as such arrays are currently being used in several different areas of proteomics and diagnostics (Bock, Coleman et al. 2004). The chemistry encompassed by two-step RNA synthesis will likely be compatible with both large-scale RNA synthesis and array printing (LeProust, Peck et al. 2010).

During two-step DNA synthesis, deprotection of the 5'-hydroxyl is accomplished using a solution of the peroxy anions, *m*-chloroperoxybenzoic acid and hydrogen peroxide (constant pH of 9.6 with an alkaline buffer). The first step of the synthesis cycle generates a phosphite triester. Upon exposure to the peroxy anion solution in the second step of synthesis, the terminal 5' carbonate protecting group is

removed, the phosphite triester is oxidized to the phosphotriester and the next round of synthesis can begin.

5.2.   Results

5.2.1.   Synthesis Cycle

Application of the two-step approach to the synthesis of RNA required minimal changes to the solid phase synthesis cycle used in two-step DNA synthesis as designed for an ABI 394 DNA/RNA synthesizer by Sierchala and co-workers (**Figure 5.1**).
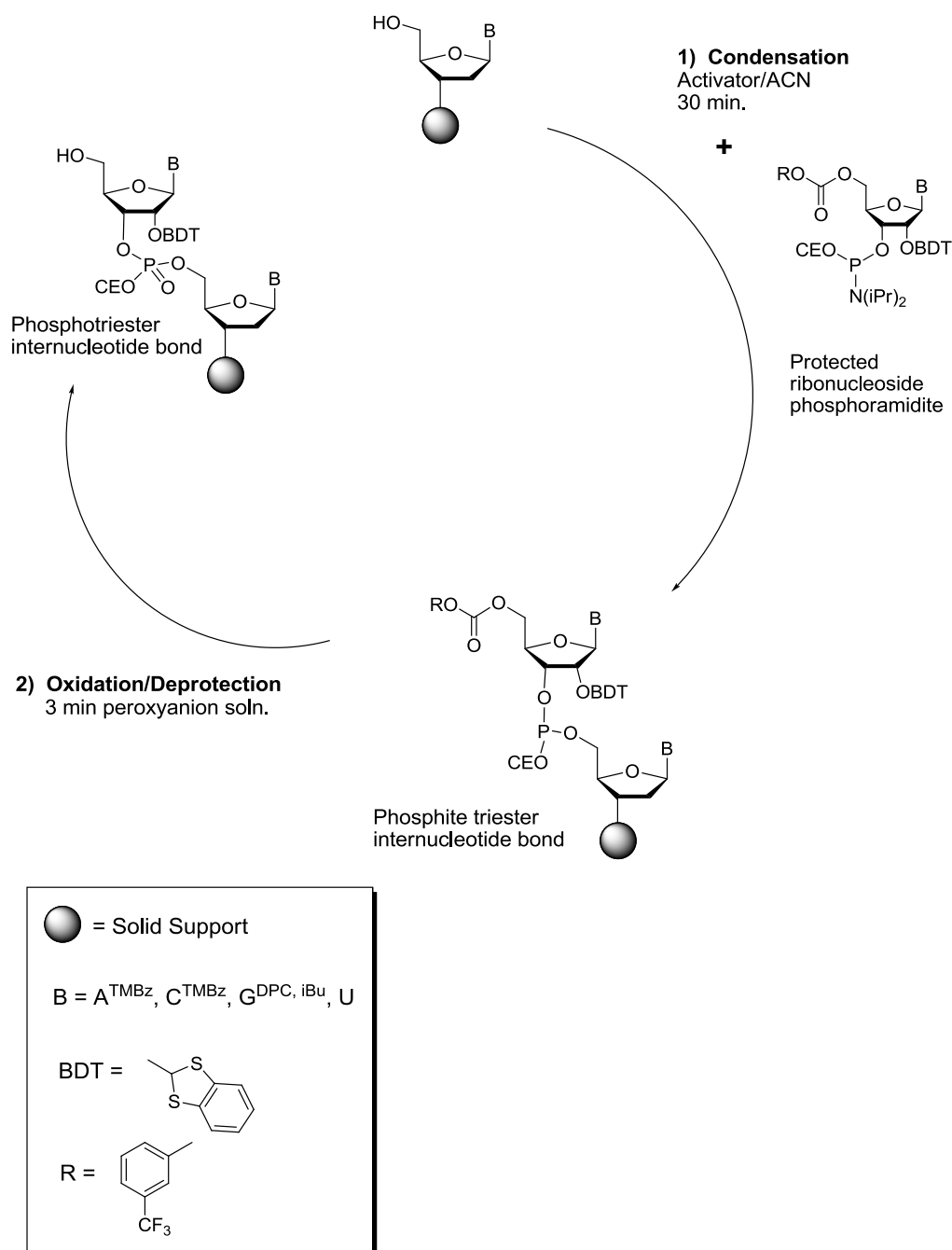
Figure 5.1: The Synthesis Cycle Utilized in Two-Step RNA Synthesis.

The only changes made to the two-step DNA synthesis cycle were the choice of activator and the coupling time used during chain elongation. Because ribonucleoside phosphoramidite monomers are known to couple at rates slower than their deoxynucleoside phosphoramidite analogs, an increase in

coupling time is typically necessary to achieve acceptable coupling yields. Therefore a coupling time of 30 minutes was used initially. For the oxidation/deprotection step, a solution of 3% (w/v) aqueous lithium hydroxide (10 mL), 1.5M 2-amino-2-methyl-1-propanol in water (15 mL), *m*-chloroperbenzoic acid (1.78 g), aqueous 30% H_2O_2 (10 mL), and dioxane (50 mL), kept at pH 9.6 was utilized. The oxidation/deprotection solution was delivered by alternating the peroxy anion solution (30 sec.) with a dioxane wash (30 sec.).

5.2.2.   Coupling Optimization

In order to analyze the coupling efficiency of the 2'-*O*-BDT phosphoramidite monomers, three different activators were tested. These activators (0.25M *S*-ethyltetrazole, 0.5M DCI and 0.25M benzylthiotetrazole) were compared by reacting 5' FARCO-2'-*O*- BDT ribouridine with polymer linked 2'-deoxythymidine using a coupling time of 30 minutes. The resulting oligomers (U$_7$T) were subsequently cleaved from support with aqueous ammonia and analyzed using reverse phase HPLC.

As can be seen in the HPLC chromatograms (**Figure 5.2**), the amount of earlier eluting failure sequences was comparable for all three; however, *S*-ethyltetrazole (**Figure 5.2**, panel C) appeared to give a superior impurity profile.
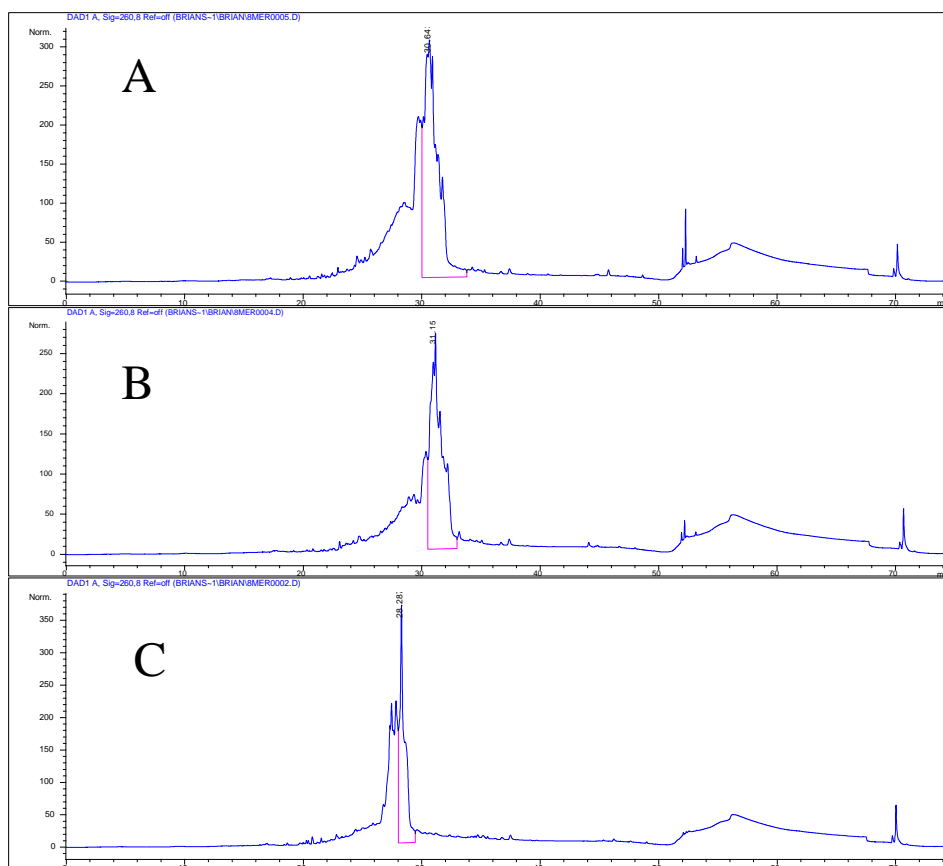
Figure 5.2: RP-HPLC Chromatograms of 2'-Protected U$_{7d}$T RNA Oligomers Synthesized Using the Two-Step Cycle with Different Activators; A: 0.5M DCI; B: 0.25M BTT; C: 0.25M ETT.

S-ethyltetrazole was therefore used as the activator for the remainder of the two-step RNA synthesis studies. Before attempting the synthesis of oligomers containing the other three bases (A, C, and G), removal of the BDT group from the oligomer was studied.

5.2.3.   2'-BDT Deprotection

To find optimal conditions for the removal of the BDT protecting group from the 2'-hydroxyl of RNA oligomers, the same U_7dT sample described above (synthesized with the SET activator) was used. This sample was divided into three aliquots, evaporated to dryness and re-dissolved into three different deprotection mixtures: 100mM acetic acid, 80% acetic acid and 100mM tetrafluoroboric acid. The deprotection was carried out at 55°C for 1 hour. **Figure 5.3**, panel A is a reverse phase HPLC chromatogram of the sample treated with 100mM acetic acid, panel B is a chromatogram of the 80% acetic acid sample, and panel C is the 100mM tetrafluoroboric acid (pH 3.8) sample (30 min). Upon immediate examination, the protected oligomer peak in the chromatogram broadened significantly.   The reason for this is unknown ,
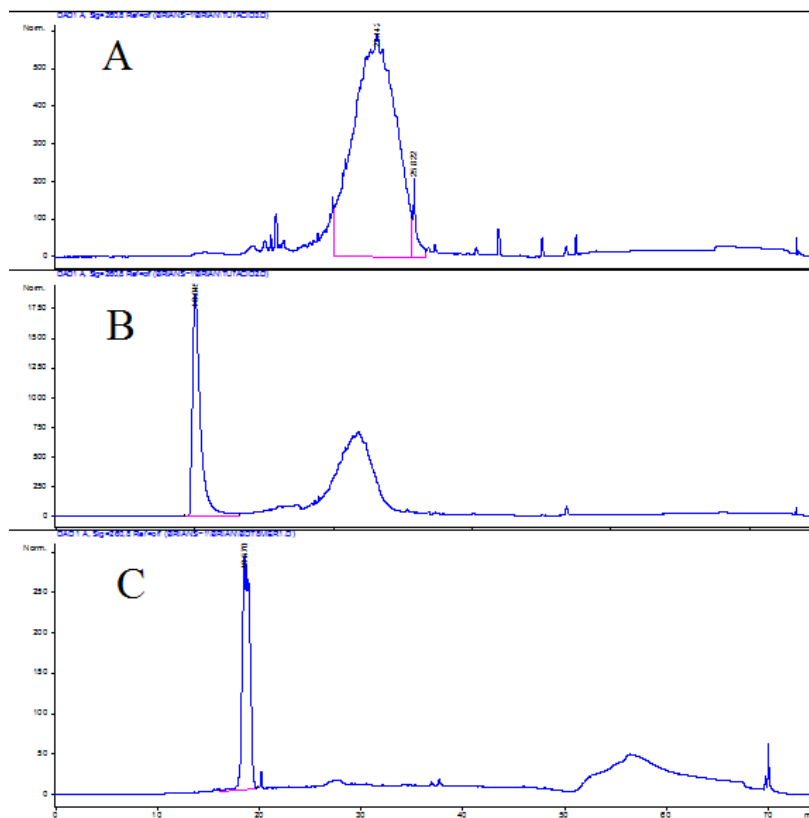
Figure 5.3: RP HPLC Chromatograms of 2'-BDT Deprotection Reactions Using Different Acidic Conditions. A: 100mM Acetic Acid; B: 80% Acetic Acid; C: 100mM Tetrafluoroboric acid.

The 100mM acetic acid sample shows little or no deprotection while the 80% acetic acid sample shows a profile consistent with some deprotection leading to degradation due to the highly acidic nature of the mixture. The tetrafluoroboric acid sample appeared to be completely deprotected after 1 hour; however, the resulting peak at ~18 min also appeared to be very broad. Analysis of this sample by infusion ESI MS showed no product peak, only a population of unknown molecular weights. Other experiments performed in the Caruthers Lab during this time hinted to the possibility of peroxy anions reacting with the uridine nucleobase (Unpublished data). Therefore, an experiment was performed to test for uridine degradation products.

A sample of 5'-DMT ribouridine nucleoside was dissolved in the peroxy anion deprotection mixture and allowed to react for 5 hours. The resulting product mixture was purified and analyzed. Infusion ESI-MS of this sample showed a peak at 517.3 in positive ion mode and a peak at 528.9 in negative ion mode. These ions correspond to the compound shown in **Figure 5.4.** This compound is a reported by-product generated by exposure of the uridine nucleobase to peroxy anions (Hill 2010).
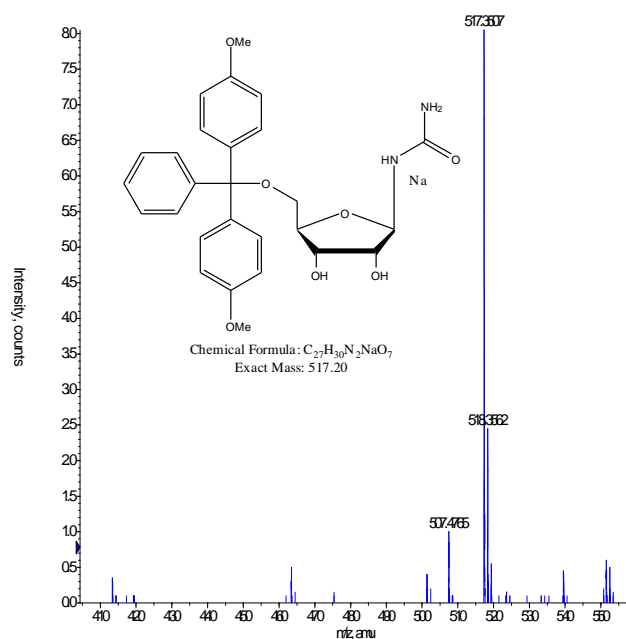
Figure 5.4: ESI-MS Spectrum of the 5'-DMT Uridine Degradation Product and Proposed Structure.

In an attempt to prevent degradation of uridine, three protecting groups were employed at the O^4 position of the uridine nucleobase: acetyl, diphenylmethylsilylethyl and 2,6-dimethylphenyl. Surprisingly, all three of these compounds were also degraded by the peroxy anion mixture. Thymidine has been shown to be stable to exposure to peroxy anions (Sierzchala, Dellinger et al. 2003). In light of these results, it was hypothesized that substitution at C^5 of uridine would impart stability, so 5-

bromoribouridine and 5-fluororibouridine were exposed to the peroxy anion mixture and both were degraded over time.

These results possibly explain the broadness of the product peak in the HPLC chromatograph shown in **Figure 5.3**, panel C and why no product ion was seen when the same oligomer was analyzed by ESI as the peak seen in HPLC chromatogram most likely represented a population of degradation products. The other nucleobases A, G and C were also exposed to the same peroxy anion conditions and no degradation was seen.

5.2.2 Solid Phase Synthesis of Oligoribonucleotides Containing A, G, and C.

In order to synthesize oligoribonucleotides containing the remaining three bases, the synthesis cycle was kept the same. Using a deoxythymdine derivitized support, the sequences A_9dT, C_9dT and G_9T were synthesized. After cleavage from the support, samples were dried, resuspended in aqueous10% acetonitrile, and a portion of the crude material was immediately analyzed using reverse phase HPLC (the 2'-hydroxyl was still protected with the BDT group)**. Figure 5.5** shows a representative RP-HPLC chromatogram of the sequence C_9dT. The chromatogram shows several peaks, indicating a failed synthesis.
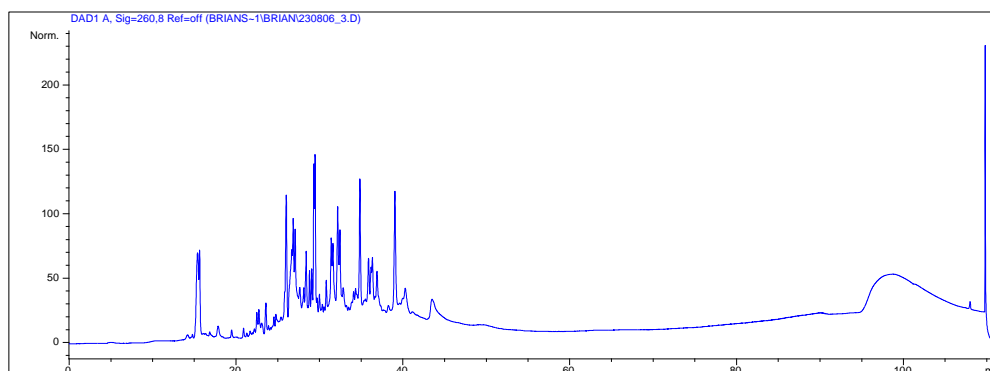
Figure 5.5: RP-HPLC chromatogram of a crude 2'-*O*-BDT protected C$_9$dT oligomer.

However, the same sample was subsequently deprotected using 100mM tetraflouroboric acid and again analyzed by reverse phase HPLC. After deprotection, the chromatogram showed a pattern consistent with that of a successful synthesis as seen in **Figure 5.6**, Panel A. Following desalting of the oligomer, infusion ESI MS showed a peak at 1492.7 consistent with the 2nd charge state of the full length oligomer (2988.8).
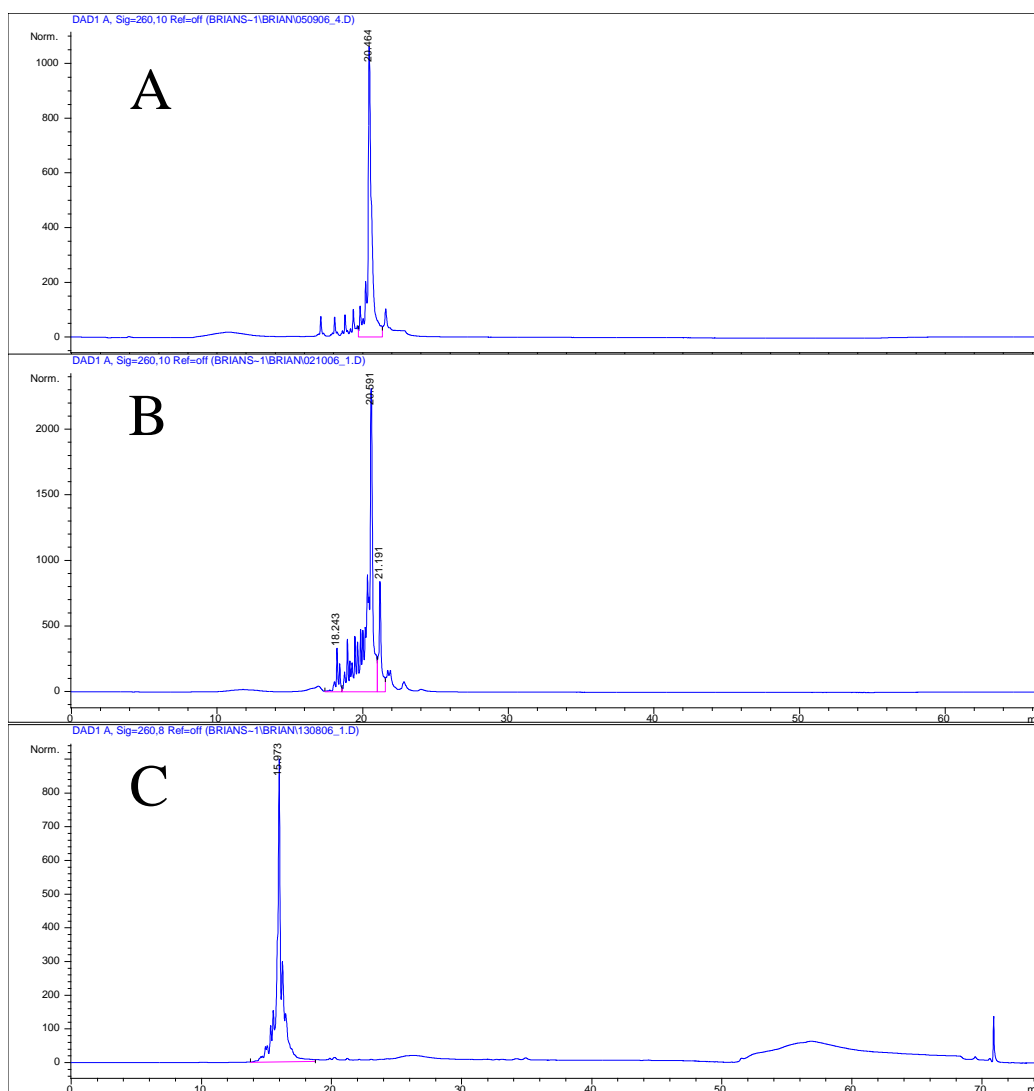
Figure 5.6: RP-HPLC Chromatograms of 2'-Deprotected Oligomers. A: C$_9$dT; B: A$_9$dT; C: G$_9$T

Chromatograms of A$_9$T and G$_{9T}$ before 2'-deprotection showed results similar to 2'-protected

C$_9$T. Therefore, analysis of synthesis fidelity of these sequences was performed after BDT deprotection

and representative chromatograms are shown in **Figure 5.6**, Panels B and C. The chromatogram of C$_9$dT

shows a late eluting peak after the main product peak. This peak is presumed to be the result of

incomplete deprotection of the nucleobase protecting groups as the amount present in the crude material

increases when only treated with ammonia for 24 hours as opposed to 48 hours. Treatment with ammonia

for more than 48 hours, however, does not decrease the amount present. Identity of the product oligomers

was confirmed using infusion ESI-MS and the data for all three oligomers is summarized in **Table 5.1**.

Sequence (5'-3')	Expected Mass $[M-2H]^{-2}$	Observed Mass $[M-2H]^{-2}$
UUUUUUUdT	1497.8	n/d
CCCCCCCCCdT	1493.4	1492.7
AAAAAAAAAdT	1601.5	1601.1
GGGGGGGGGdT	1673.5	1674.3

Table 5.1:   **Expected and observed ESI MS data for the fully deprotected RNA oligomers.**

5.3.   Discussion

Using the protected ribonucleoside phosphoramidites described in the previous chapter, the two-

step synthesis cycle appears to be compatible with solid phase RNA synthesis. Despite the apparent

instability of the uridine nucleobase to peroxy anions, further work needs to be performed to find an

appropriate protecting group compatible with the two-step chemistry. Reports suggest a possible

mechanism for this degradation involving nucleophilic attack by a peroxy anion on C^6 of uridine, leading

to epoxidation of the C^6-C^5 double bond. Further hydrolytic cleavage leads to the product shown in Figure

5.4. The stability of thymidine to peroxy anions could therefore be explained by an inability to epoxidize

the C^6-C^5 bond due to ring strain or steric interference introduced by the methyl group at C^5. However, the exact mechanism is unknown due to an inability to isolate any reaction intermediates.

In light of this, a possible solution to the nucleophillic attack at C^6 if uridine could be the use of an electon donating group at the O^4 position of uridine. For example, the diphenylcarbamoyl may provide enough electron donation to the C^6 carbon to prevent Michael addition at that position. The stability of thymdine to peroxyanions shows that simply a methyl group provides stability to the chemically similar nucleobase uridine. Alternatively, lowering the pH of the peroxyanion solution may be an alternate solution to the uridine degreadation problem. Doing a proper ranging study of pH along with time necessary to deprotect the 5'hyroxyl group would certainly be worthwhile.

Fortunately, the nucleobases of A, G and C proved to be stable to the peroxy anion mixture. The 5'-FARCO-2'-BDT ribonucleoside phosphoramidites appeared to be sufficiently reactive for use in the solid phase, two step synthesis approach. Accurate coupling efficiencies could not be calculated due to a lack of a capping step during synthesis; however, the profile of failure sequences is acceptable at this stage of the chemistry. The reverse phase HPLC chromatograms (**Figure 5.5**) of the 2'-protected oligomers is still unexplained. It could be hypothesized that aromaticity of the BDT group is somehow aiding in aggregation of the oligomers or more likely, the sulfur atoms on the BDT group may be oxidized to a variety of states throughout the oligomer resulting in a population of compounds before 2' deprotection. This hypothesis would be consistent with the deprotection reaction resulting in a sharper product peak in RP-HPLC. Further analysis would be required to understand the heterogeneous profile observed in **Figure 5.5**, but it is clearly correlated to the BDT group.

5.4.   Materials and Methods

5.4.1.   General Procedures

All DNA/RNA synthesis reagents including activators, solid support columns, and Poly-Pak II columns were purchased from Glen Research (Sterling, VA). The solid phase synthesis of all oligomers was performed on an ABI 394 DNA/RNA synthesizer acquired from Applied Biosystems (Foster City, CA). All solid phase syntheses were performed on a one micromole scale and the cycle was adapted from the published two-step synthesis cycle previously published in the Caruthers' Lab (Sierzchala, Dellinger et al. 2003) with the coupling time increased to 30 minutes. Phosphoramidite monomers (0.1M in anhydrous acetonitrile), activator (0.25M SET in anhydrous acetonitrile) and peroxy anion solution were all prepared immediately prior to use.

After each synthesis, CPG columns were washed with anhydrous acetonitrile, dried with argon and placed in a one dram vials. Concentrated ammonia was added and each vial then sealed with a Teflon lined screw cap. The vials were placed in a 55°C heating block and allowed to react for 48 hours. Following the cleavage reaction, each vial was cooled on ice, the CPG removed by filtration, and the supernatant evaporated to near dryness *in vacuo*. BDT deprotection reactions were performed by resuspending oligomers in either 100mM acetic (adjusted to pH 3.8 with TEMED), 100mM tetrafluoroboric acid (adjusted to pH 3.8 with TEMED) or 80% acetic acid and heated to 55°C for one hour. Desalting of oligomers prior to ESI-MS analysis was performed using Poly-Pak II according to procedures supplied by the manuacturer.

5.4.2.   RP HPLC Analysis of Oligomers

Crude oligomers were analyzed on an Agilent 1100 series HPLC using a Hypersil ODS 4.0 X 250mm C-18 column. Eluents were: (A), 100mM triethylammonium acetate in water, pH 7.5; (B), Acetonitrile. The eluent gradient was 0-600% B in 42 minutes at a flow rate of 1.0 mL/min while observing at wavelength 260nm.

5.4.3.　　ESI-MS Analysis of Oligomers

ESI MS data were obtained using an ABI Pulsar Q-Star Q-TOF spectrometer by infusion in pure water.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

6.1.   Phosphonoactete RNA

An RNA analogue such as phosphonoacetate RNA has much promise in the area of antisense, RNAi and aptamer therapeutics. This is because analogs in this DNA series have been shown to be stable toward nucleases and ester hydrolysis which leads to enhanced transfection (Yamada 2003) (Yamada, Dellinger et al. 2007) (Dellinger, Sheehan et al. 2003). In attemps to synthesize phosphonoacetate RNA, initial experiments indicated that condensation yields varied from   to   and these yields were dependent upon the type of protection used for the 2'-protecting group. For example with the   , yields were acceptable at % whereas with   , the yields per condensation were much less (%). However serious problems were encountered during removal of 2'-protecting groups. For example with the ACE protecting group, the basic conditions required in the second step of deprotection lead to cleavage of the internucleotide linkage. Both mass spectral data and HPLC patterns showed massive degradation. Furthermore the presence of nucleoside 2'-*O*-phosphonoacetate monoester indicated that transesterification occurred during deprotection like following the mechanism in **Figure 6.1**.
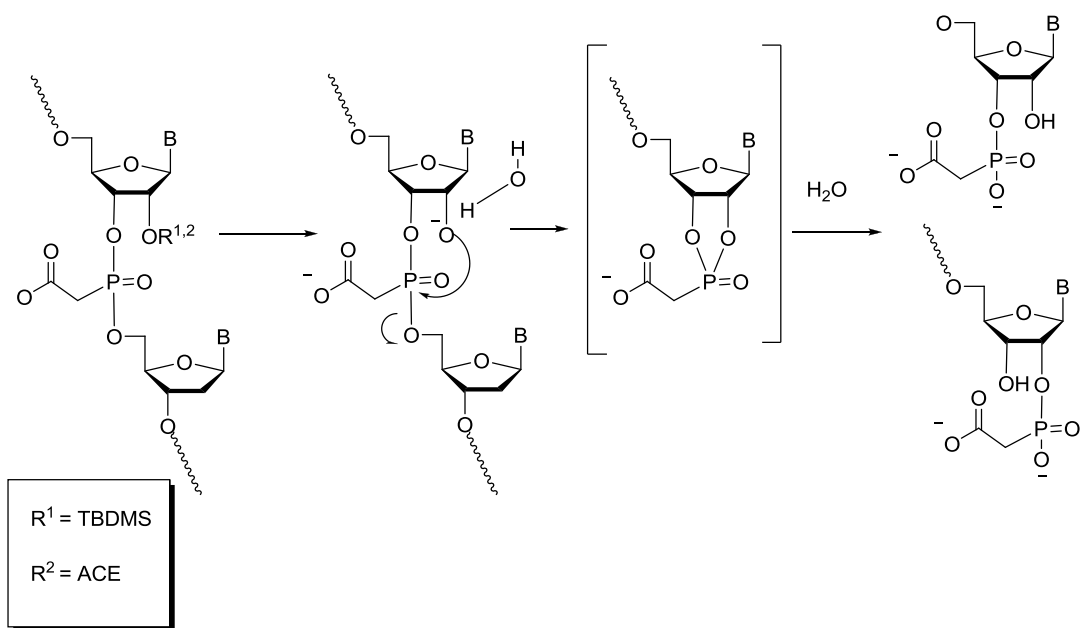
Figure 6.1: Proposed mechanism for cleavage of the phosphonoacetate linkage in aqueous conditions.

However, a study looking at a pH range during the 2'-deprotection step could lead to a successful phosphonoacetate oligomer.

When 2'-*O*-TBDMS or 2'-*O*-TOM groups were used to protect the 2'-hydroxyl, the internucleotide linkage was also unstable upon treatment with TBAF (universally used to remove silyl protecting groups). Other fluoride ion sources such as TEA:3HF and pryridinium hydroflouride should have been examined and the pH upon treatment should also have been monitored by diluting a portion of the organic solution into water and taking a pH. This doesn't result in the actual pH but does allow for a relative pH range to be examined. A milder solution of fluoride may have been successful in removing the silyl group without strand cleavage due to transesterification.

The use of a 2'-*O*-ACE or other acid labile groups also should be re-examined with more thorough attention to pH, time and temperature during deprotection. Simple experiments using a solution of acetic acid at a pH range of 3-6 should have been designed. This sample set should have then been cut into two or three temperatures between 20-55ºC taking time points every 30 minutes (neutralizing them before analysis). Reversed phase HPLC analysis would most likely have shown a trend in the stability of the phosphonoacetate linkage to acid treatment with a vicinal hydroxyl.

New and exciting 2'-protecting groups for RNA synthesis have recently been discovered and used successfully to make RNA oligomers in high fidelity (Dellinger, Timár et al. 2011). These groups such as the thionocarbamate should be examined (**Figure 6.2**).
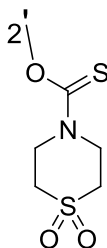
Figure 6.2: Structure of the 2'-*O*-thionocarbamate protecting group.

The 2'-*O*- thionocarbamate group is removed using ethylenediamine under non-aqueous conditions. In organic solvents it is known the pKa of weak acids such as a 2'-hydroxyl increase, thus the deprotected 2'-hydroxyl is less likely to be deprotonated by an amine (Sarmini and Kenndler 1999). Using this methodology would decrease the oxyanionic attack of the 2' hydroxyl of the phosphonoacetate linkage and possibly allow for a stable oligomer following 2' deprotection.   In aqueous conditions the oxyanion is stabilized by an H_2O proton (see **Figure 6.1**). Eliminating this stabilization decreases nucleophilicity of the vicinal oxyanion. Avoiding such aqueous conditions utilizing the thionocarbamate protection at the 2'-hydroxyl may prevent strand cleavage

6.2. 2-Step Cycle for RNA Synthesis

Simultaneous oxidation and 5'-deprotection of a DNA oligomer offered many advantages relative to many DNA synthesis protocols. When attempting to synthesize RNA on a solid support, especially longer oligomers, iterative treatment with acid can result in depurination. Also because of the reversibility of the DMT cation without constant flow of acid results in incomplete deprotection. This would be the challenge on chips and for large scale synthesis of RNA. Thus the idea of using a two-step process free of acid, fewer chemical reactions, and an irreversible 5' deprotection step was therefore very promising in translating this chemistry to RNA synthesis.

Using the-step cycle in combination with the BDT protecting group, RNA oligomers were successfully made. Poly A, C, and G oligonucleotides were synthsesized on solid support and their identities confirmed using LC/MS.

The result that uridine was extremely susceptible to peroxy anion degradation was unfortunate.   Over the past few years the fact that uridine is susceptible to other nucleophiles such as alkylamines has been reported (Hill 2010). Further research into other orthogonal protecting groups will be necessary to keep uridine in tact using a two-step approach to RNA synthesis. One possible solution would be to use 5-methyl uridine instead of uridine. The methyl group adds electron donation, as well as steric hinderance which lends the 6 position of the pyrimidine base less susceptible to nucleophiles.

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