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Deoxy-Amine Nucleoside Polymers (dANPs) to Control Gene Expression

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Abstract

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This study aims to explore the *de novo* synthesis of deoxy-amine nucleoside polymers (dANPs) that function as antisense oligonucleotides. The synthetic route requires 8 steps starting from thymidine. Template-directed ligation will be used to polymerize monomers into chains that can be 32 base pairs long. To confirm the cellular uptake of dANPs, fluorescent labeling will be used to monitor its location within the cell. To achieve maximum yields in the synthesis, alternative pathways are tested to overcome low reactivity in certain steps.

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I. Introduction

Within the past few decades, synthetic oligonucleotides have seen significant advancements in both design and application[1,2]. Synthetic oligonucleotides are short, single-stranded DNAs that are usually less than 50 nucleotides long. These oligonucleotides can follow rational designs to be chemically modified to contain certain desired characteristics. The characteristics can include solubility, stability, and activity in cells[3]. Synthetic oligonucleotides have a wide array of applications, ranging from therapeutics to the study of gene function. Fomivirsen, a 21 nucleotide long antiviral drug was approved by the FDA in 1998 for the treatment of cytomegalovirus retinitis (CMV), an infection that causes inflammation of the retina in the eye[4]. More recently, the FDA approved Mipomersen as a cholesterol-reducing drug in January, 2013[5]. The concept behind the use of synthetic oligonucleotides is relatively simple. The sequence of a target gene of interest is identified for the design of the synthetic oligonucleotide. The full sequence of the gene, however, does not have to be determined as the designed oligonucleotide is complementary only to a short stretch of bases. Studies suggest that the minimum sequence that is likely to be unique within a specific mRNA pool is around 13 bases, so any sequence that contains 13 or more base pairs can be considered specific[6]. Hybridization of the synthesized oligonucleotide with the target gene sequence will downgrade or eliminate expression of a gene through transcriptional splice blocking[3,7], translational steric blocking[7], or RNAse H dependent degradation[2].

There are several ways to achieve regulation of gene expression, but all of them require the synthetic oligonucleotide to hybridize with the target DNA or mRNA sequence. The first mechanism involves regulation at the level of transcription. In a eukaryotic cell, RNA splicing is involved in modification of the pre-mRNA by intron removal. The remaining exons are joined

together to form the functional mRNA product that can be translated into a protein. A synthetic oligonucleotide can be designed to be complementary to the site of splicing so that part of the strand hybridizes with an exon while the other half of the strand extends into an intron. This base pairing prevents the proper inclusion of exons that would originally have been present in the final mRNA product, which can lead to nonsense or varied gene product (Figure 1). The other mechanism acts at the translational level. During translation of an mRNA to protein, the ribosome binds to the ribosomal binding site (RBS) and then continues to translate the RNA sequence to a protein product. When a short strand of complementary sequence binds to the RBS or the beginning of the mRNA, the ribosome will be unable to translate the mRNA due to steric hindrance. The gene expression of a cell affected by this mechanism can be down-regulated or eliminated according to the binding affinity of the synthesized sequence. One more mechanism involves the utilization of RNAse H, which is an endonuclease that can cleave RNA sequences. Although the precise mechanism of RNAse H recognition is not fully known[8], a sequence with properties similar to those of DNA can activate RNAse H degradation. The advantage of down-regulation by this mechanism is that binding of the synthetic oligonucleotide to any location of the target mRNA can induce degradation with up to 80%-95% effectiveness[2]. In contrast, down-regulation by steric hindrance requires the sequence to target specific sites on the target gene, namely the beginning of the 5' end.

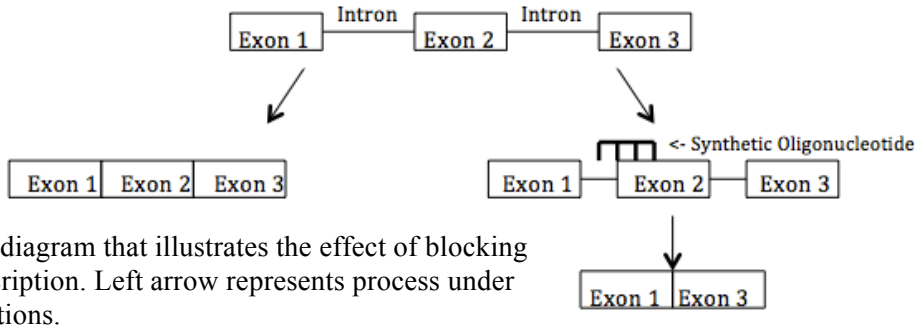


Figure 1. A diagram that illustrates the effect of blocking normal transcription. Left arrow represents process under normal conditions.

The use of synthetic oligonucleotides for the treatment of a disease or genetic disorder is termed antisense therapy, leading to the field of antisense technology. The first major breakthrough with antisense technology in medicine was in 2006, when Warfield and colleagues successfully protected macaques from Ebola viral infection[9]. The breakthrough was very successful as 75% of patients recovered from the infection. This finding, along with the FDA approved antisense drugs, paved the way for a therapy that is still developing.

Currently, there are numerous oligonucleotides that are synthesized with distinct functionalities. The first synthetic oligonucleotide dates back to 1967 where the Belikova group prepared natural DNA compounds[10]. A decade later, Stephenson and Zamecnik investigated a tridecamer that effectively inhibits translation of proteins by the Rous sarcoma virus by competitive hybridization[11]. These oligonucleotides, though synthetic, are natural DNA compounds with natural functional groups. As time progressed, other researchers have investigated a variety of functional groups to observe changes in oligonucleotide characteristics. One of the earliest modifications was changing the phosphodiester bond to a methylphosphonate[2]. The methylphosphonate is relatively stable under biological conditions and is neutrally charged. The absence of charge however, reduces the solubility and uptake by the cell which is believed to enter through endocytosis[2]. The advantage of this oligonucleotide is that

the absence of charge eliminates repulsion with the target DNA sequence during hybridization, which increases the binding affinity to target sequence[2]. The methylphosponate cannot activate the RNAse H endonuclease. Another synthetic oligonucleotide called phosphorothioate[2] replaces the nonbridging oxygen with sulfur. This replacement grants increased nuclease stability as well as the ability to activate RNAse H endonuclease[2]. However, the introduction of a sulfur group creates a helix destabilizing hindrance, which manifests as a lower melting temperature. Another problem with the phosphorothioate is nonspecific binding of other cellular proteins.

Current "second generation" oligonucleotides are more resistant to degradation by nucleases and hybridize with higher affinity and specificity[2]. They include morpholino oligonucleotides, PNA, and LNA. Most of these oligonucleotides, however, maintain the phosphate backbone and have negative or neutral charge. Some criteria to consider when designing synthetic oligonucleotides include stability, affinity, cellular uptake, solubility, toxicity, and specificity. Stability is important because the synthetic oligonucleotide has to reach its target intact to be effective. If during delivery it degrades, then it cannot hybridize with the target strand and regulate gene expression. This problem can be solved by modifications such as those of phosphorothioate. Cellular uptake is one of the most important factors to consider, because studies have shown that although some modified antisense oligonucleotides can penetrate the cell membrane, the intracellular concentrations are not sufficient to elicit pharmacological activities[1]. Many groups utilize "synthetic vectors" which can protect the nucleic acid while delivering the cargo inside cells. These vectors can be cationic or anionic lipids, which facilitate the passage through the plasma membrane[12]. However, cationic liposomes have been shown to be toxic to some cultured animal cells[13].

Deoxy-amine nucleoside polymers (dANPs), designed in our lab, replace the traditional phosphate backbone with an amine backbone through imine condensation and subsequent reductive amination. This synthetic oligonucleotide is predicted to have a better affinity through the interaction between the positive charge and the negatively charged target DNA molecule. In addition, dANPs do not have the usual phosphate backbone (Figure 2), which can avoid recognition by intracellular nucleases. By taking advantage of its stability and predicted cellular uptake, the dANP is a perfect platform for antisense therapy which other synthetic oligonucleotides lack. They can also be used for studies of gene functions in model systems.

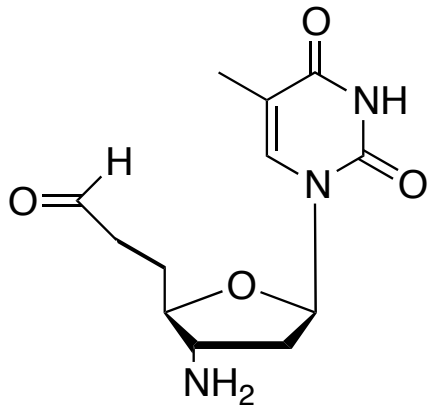


Figure 2. A single monomer of the dANP.

II. Experimental

General Methods. Reactions were run in 25mL, 100mL, or 250mL round-bottom flasks with magnetic stir bar, under no protection, unless otherwise noted. All solvents used were of highest purity anhydrous solvents from EMD Millipore DriSolv®. Reagents were from Sigma-Aldrich unless otherwise noted. Reactions were followed by standard workup procedures using ethyl acetate, water, and brine. Subsequent flash column chromatography was used to purify product when necessary. Aluminum thin layer chromatography (TLC) plates were used to monitor both the progress of reaction as well as product separation during column chromatography. Nuclear magnetic resonance (NMR) imaging was used to determine purity as well as to verify the composition of obtained products. Mass spectrometry was also used to further confirm product composition.

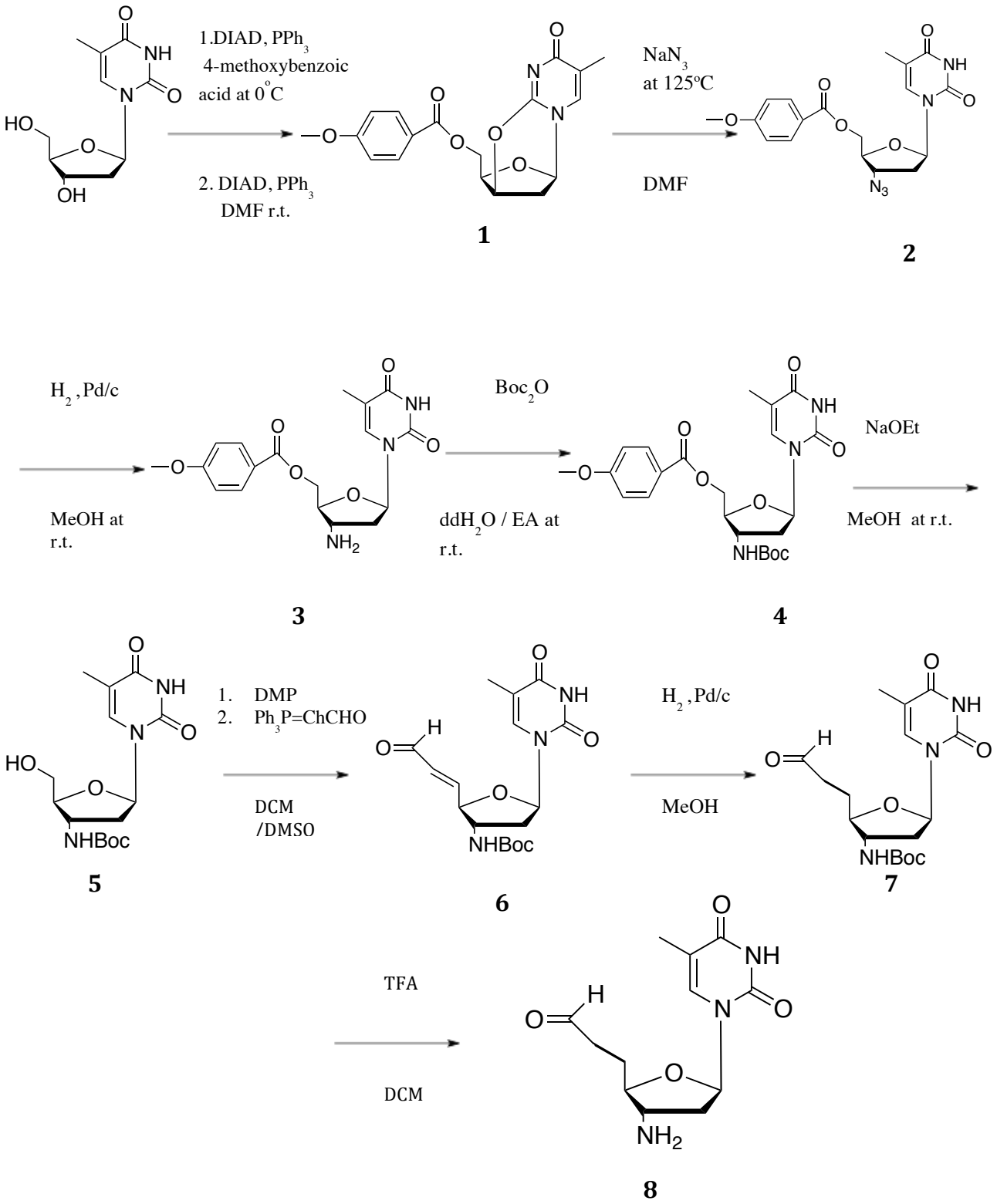
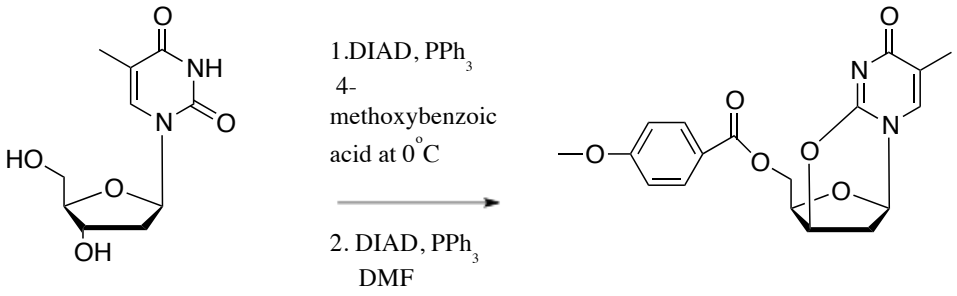
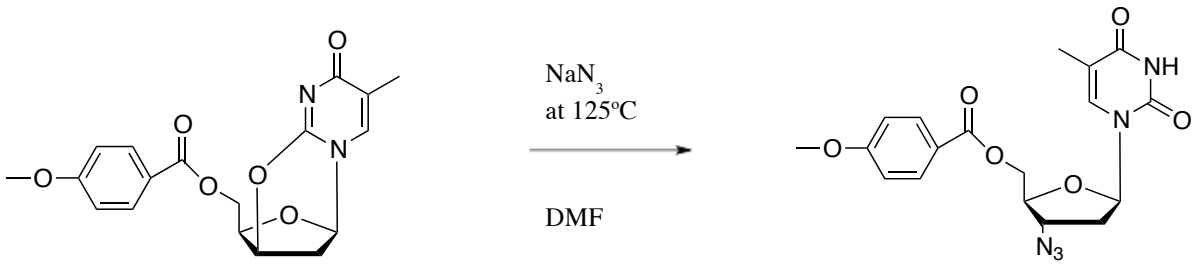


Figure 3. Synthetic route of the dANP monomer.



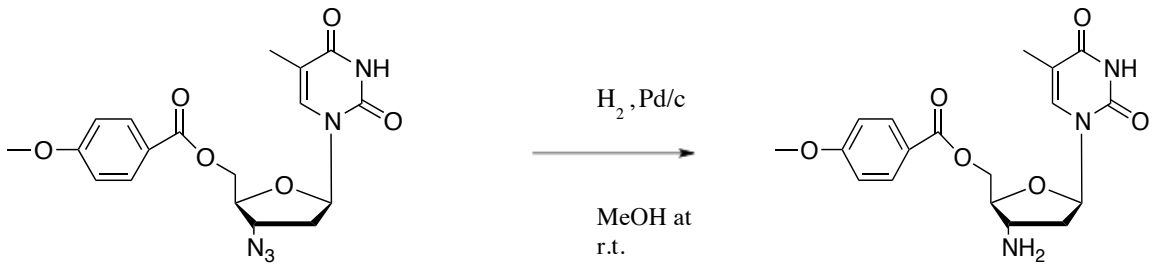
1

To a 250mL round-bottom flask, thymidine (5.00g, 20.6 mmol) and triphenylphosphine (8.10g, 1.50 eq) was dissolved in DMF at 0°C. 4-methoxybenzoic acid (4.70g, 1.50 eq) and DIAD (6.1 mL, 1.50 eq) was stirred in a 100mL flask and added drop-wise to the solution over 20 minutes. The solution turned light yellow after 30 minutes of stirring. Triphenylphosphine (8.10g, 1.50 eq) was added to the solution and DIAD (6.1 mL, 1.50 eq) was added drop-wise over 20 minutes at room temperature. The solution was allowed to react over 1 hour. 400 mL of diethyl ether anhydrous was pre-chilled in a 500mL Erlenmeyer flask. Solution was poured into diethyl ether to allow precipitation of product. White solid was collected by gravity filtration.



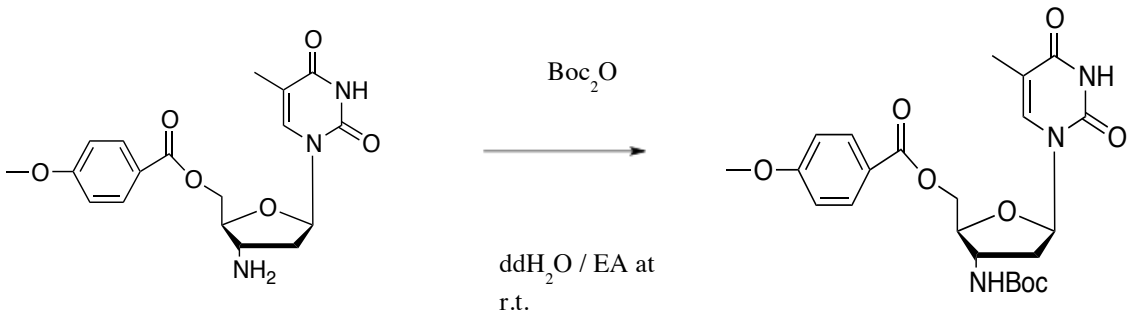
1 **2**

To a 250ml round-bottom flask, compound **1** (5.10g, 15.6 mmol) and sodium azide (1.39g, 1.50 eq) was dissolved in 50mL DMF. White solids were visible and could not be fully dissolved. Solution was allowed to react at 125°C over 6 hours. White precipitate was filtered by gravity filtration and the solution was added to a separatory funnel. 150mL of 5% HCl/water solution was added to the funnel to generate a white, milky solution. Ethyl acetate was used to extract the aqueous phase 5 times and the organic phase was subsequently combined. The organic phase was then washed with brine 5 times and dried over $MgSO_4$ for 15 minutes. The solution was filtered through gravity filtration to remove $MgSO_4$. Solution was dried with rotary evaporator and yielded an orange, soft solid.



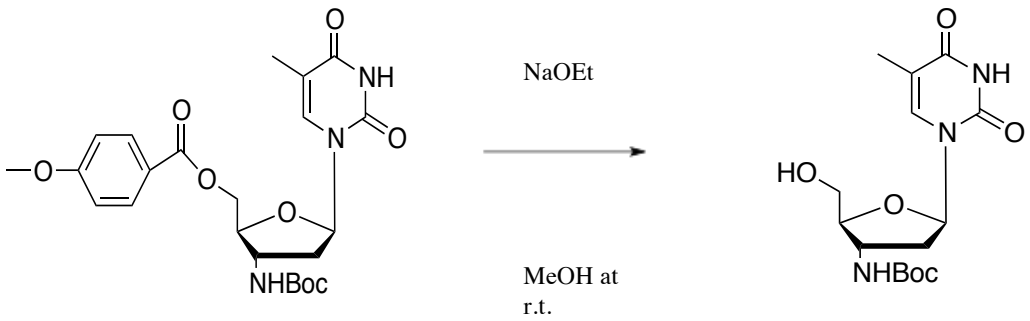
2 **3**

To a 250mL round-bottom flask, compound **2** (4.01g, 9.99 mmol) was dissolved in methanol and palladium on activated carbon (5% w.t.) was added. The flask was purged repeatedly with H_2 and connected with a hydrogen-filled balloon overnight. Carbon was removed by gravity filtration and solution was dried with rotary evaporator. Column chromatography (EA:methanol = 10:1) was applied to elute the main product near the bottom of the TLC plate. The end product was a white, yellow solid.



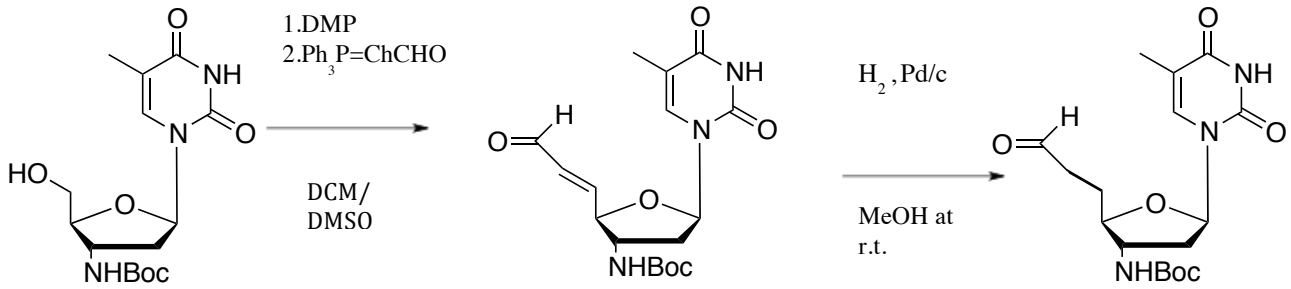
3 **4**

To a 250mL round-bottom flask, compound **3** (0.90g, 2.39 mmol) and tert-butyloxycarbonyl (Boc) (0.66g, 1.20 eq) was dissolved in 25mL ethyl acetate and 25mL ddH_2O. Solution was allowed to react overnight at room temperature. Solution was dried with rotary evaporator to yield a white solid.

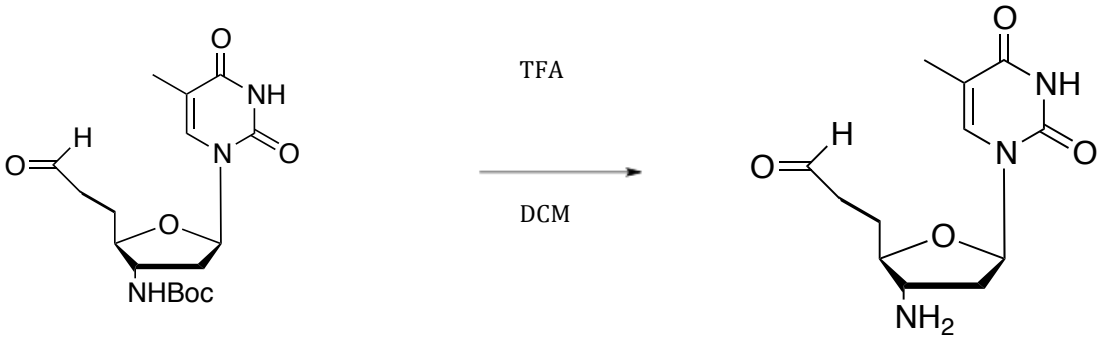


4 **5**

To a 250mL round-bottom flask, compound **4** (1.1g, 2.30 mmol) and sodium ethoxide (21% solution) was allowed to react in methanol at room temperature overnight. Column chromatography (EA: hexane = 1:3 , EA: hexane = 3:1) was used to elute the second dot on TLC plate. The product was a white solid with hints of orange if impure.

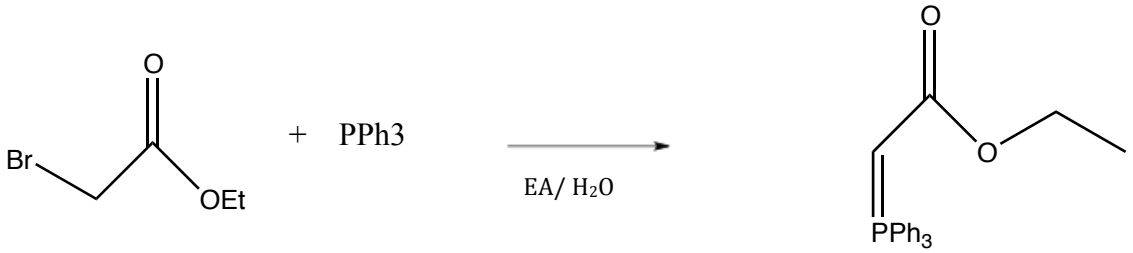


To a 100mL round-bottom flask, compound **5**(100mg, 0.29 mmol) and Dess-Martin periodinane (188mg, 1.50 eq) was dissolved in dichloromethane. 1.5 mL DMSO was added drop-wise to solution. Solution was allowed to react over 2 hours at room temperature. White precipitate appeared after 2 hours. (Triphenylphosphoranylidene) acetaldehyde (134mg, 1.50 eq) was added to the solution and it was stirred overnight. White precipitate was filtered by gravity filtration and brine was used to wash out excess DMSO. Solution was dried with rotary evaporator and redissolved in methanol. Palladium with activated carbon (5% w.t.) was added to solution and the flask was purged repeatedly with H_2. The flask was allowed to stand overnight. Column chromatography (EA:hexane =3: 1) was used to elute the first major product.



7 **8**

To a 25mL round-bottom flask, compound **7** was dissolved in 2mL dicholoromethane. 1mL of trifluoroacetic acid was added drop-wise to solution and allowed to react at room temperature over 1 hour. 1mL of ddH_2O was added to solution when reaction completed as indicated by TLC. Solution was adjusted to pH of 7 with ammonium oxide. Dichloromethane was dried with rotary evaporator. Ethyl acetate was used to wash the aqueous phase to remove impurities. Product was dried by flash freezing.



Ethyl bromoacetate (carbethoxymethylene)triphenylphosphorane

To a 250mL round-bottom flask, triphenylphosphine (4.00g, 15.2mmol) was dissolved in 80 mL ethyl acetate. 1.79 mL of ethyl bromoacetate was added and solution was allowed to react at reflux (77.1^{o}C) overnight. White precipitate was collected by gravity filtration and redissolved in 24 mL dicholormethane. 130 mL of ethyl acetate was added to give white precipitate.

III. Results

I have completed the synthesis up to compound **5** of the synthetic route. The NMR and mass spectrometry data of compound **5** is shown below.

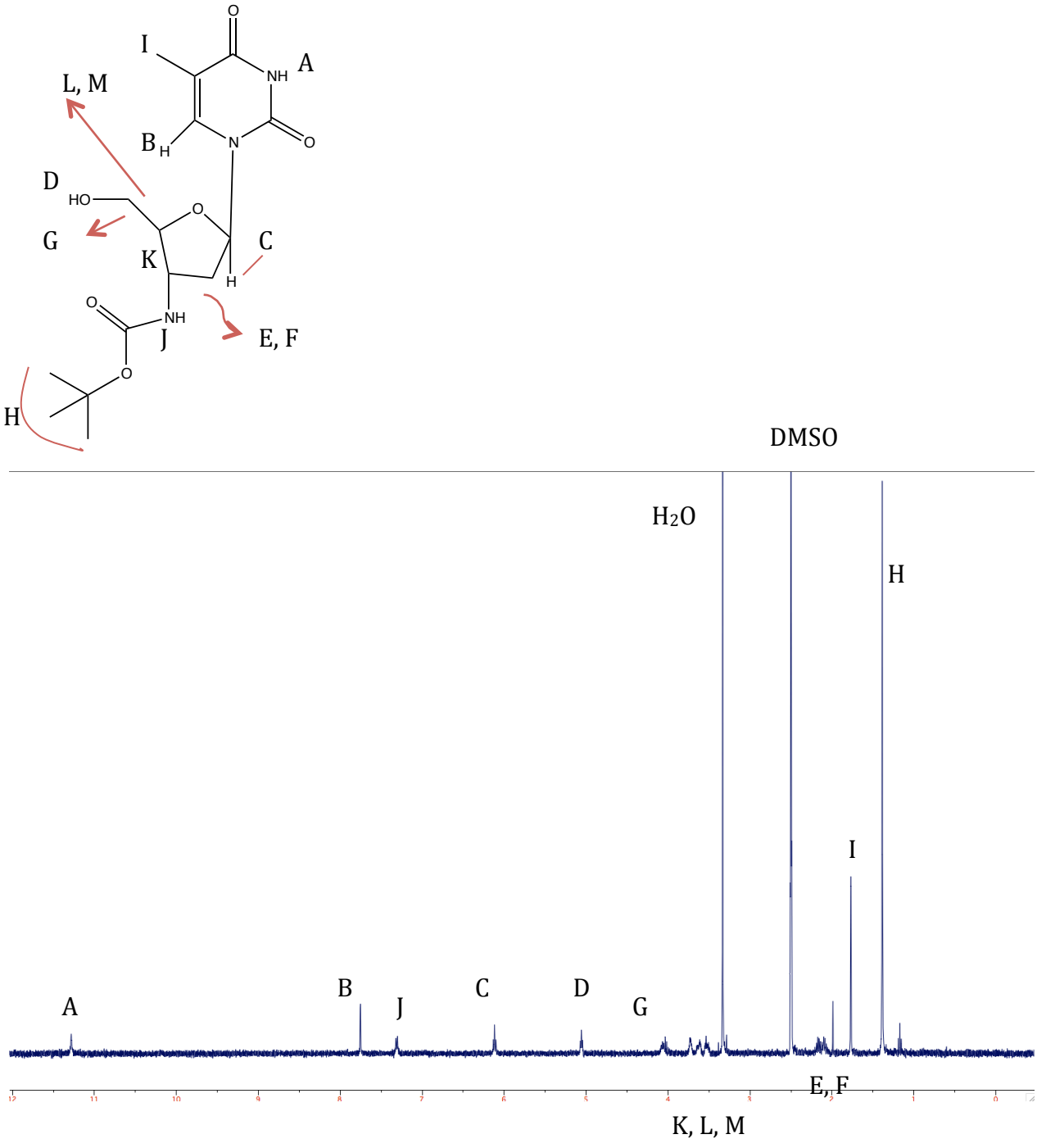
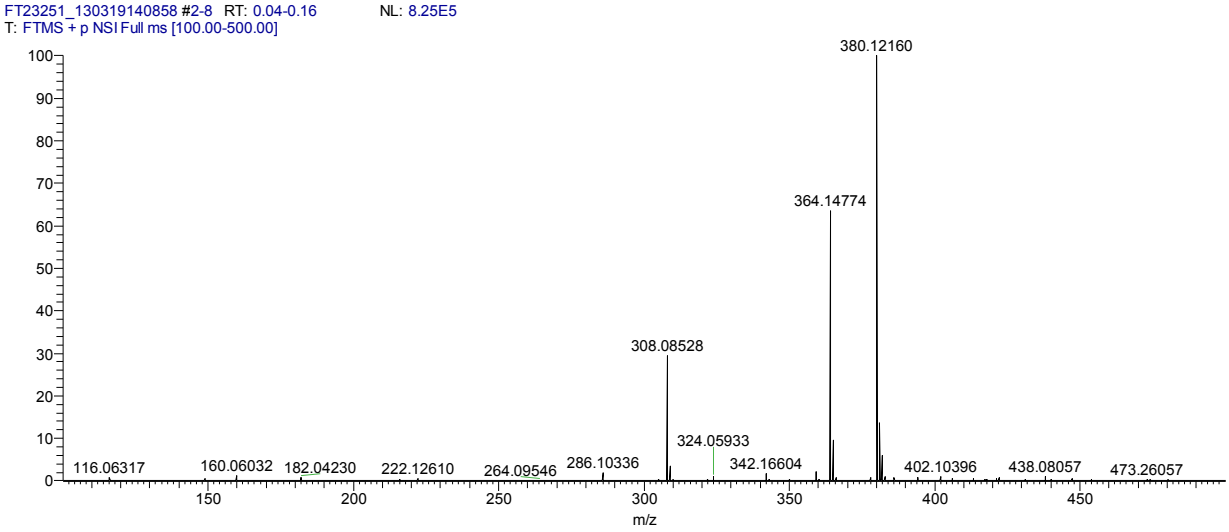


Figure 4. NMR data of compound 5. Peaks confirm the identity of the desired product.



Elemental composition search on mass 364.14774

m/z= 359.14774-369.14774

m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
364.14774	364.14791	-0.17	5.5	$C_{15}H_{23}O_6N_3Na$
	364.14924	-1.50	10.5	$C_{16}H_{19}O_2N_7Na$

Elemental composition search on mass 380.12160

m/z= 375.12160-385.12160

m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
380.12160	380.12184	-0.24	5.5	$C_{15}H_{23}O_6N_3K$
	380.12318	-1.58	10.5	$C_{16}H_{19}O_2N_7K$

Figure 5. Mass spectrometry data of compound 5. Peaks of 364.14774 and 380.12160 are both of the desired product.

IV. Discussion

Rationale for starting material and synthetic route

Thymidine was chosen as the starting material for this synthetic route for a few reasons. First of all, pyrimidines are easier to work with compared to purine bases because substituted purines tend to have low solubility in water and organic solvents. In addition, thymine does not require protection for the synthetic scheme, so this helps to avoid the need for additional steps that might complicate the synthesis. Choosing protecting groups that can selectively protect desired positions can be troublesome. Thymidine also allows the use of the Mitsunobu reaction as the first step, which selectively protects the 5' –OH group while forming an intramolecular ring. The release of ring-opening through nucleophilic attack of azide directs the desired stereochemistry. The Mitsunobu reaction also proceeded with a high yield of 93%. The protecting groups 4-methoxybenzoic acid and tert-butyloxycarbonyl (Boc) were chosen because they can selectively protect the –OH and NH_2 respectively, as well as requiring different pHs to be removed. Because 4-methoxybenzoic acid requires a strong base to remove, while the Boc group requires a strong acid, this separation allows deprotection to be controlled separately when desired. From compound **5** to compound **6**, a small volume of DMSO was added to help increase solubility to dissolve Dess-Martin periodinane[14]. Previous trials have shown that solubility is low if only dichloromethane was added.

Oxidation reactivity

The final reactions from compounds **5** through **8** were difficult to execute efficiently. Because of the mild conditions and relatively quick reaction rates[15], Dess-Martin periodinane was originally chosen as the oxidizing agent in this reaction. However, the reaction with Dess-

Martin periodinane did not run to completion as evidenced from TLC. This suggests that either the starting material was impure or the Dess-Martin periodinane was not effective. ^{1}H NMR and mass spectrometry data (Figure 4 and 5) suggested that the starting material was present and at least 90% pure. The problem then turns to the possibility of the reagent being spoiled. Schreiber and Meyer reported that laboratory with humidities of 65-75% required care to avoid "adventitious moisture" and 75% humidity quickly destroyed the reagent[15]. Taking into the consideration of the weather in Atlanta, Georgia, this not impossible. To determine whether the reagent was effective or ineffective, a simple reaction with benzyl alcohol and Dess-Martin periodinane was used to test the effectiveness of the reagent. Because Dess-Martin periodinane oxidizes primary alcohols to aldehydes, benzyl alcohol was used to determine if it oxidizes to benzaldehyde. After the reaction was completed, co-spotting the product with benzaldehyde showed that the reagent was effective. An alternative oxidation reaction was explored using the Swern oxidation. This reaction requires more attention to conditions, which is done at -78^{o}C as well as under N_2 protection. Nevertheless, Dr. Jay T. Goodwin has successfully used this reaction to form 7'-aldehydes previously[19], so this is a safe alternative.

The reaction using (triphenylphosphoranylidene) acetaldehyde also proceeded with minimal yields, making impossible for the recovery of product. A colleague currently working on a similar geometry reported no more than 30% yields. With mg scale of the reaction, the products can be easily lost due to adsorption to glassware. As an alternative, I synthesized (carbethoxymethylene) triphenylphosphorane as a substituting Wittig reagent. Saha et. al. used this reagent in their synthesis of 7'-aldehydes on thymidine with yields up to 80%[16]. With this reagent, I hope to achieve better yields to help accumulate more monomers.

Template-directed ligation

After the synthesis of the monomers, template-directed ligation will be used to polymerize them into chains. Template-directed ligation uses a complementary DNA strand as a template to allow interaction between the 3' and 5' end of the synthesized monomers. The Watson-Crick base pairing of the monomers and template strand brings the functional groups in close proximity. Under polymerization conditions, the functional groups will react through imine condensation (Figure 6). Subsequent reductive amination will irreversibly trap the product as secondary amine. The advantage of using the template-directed ligation can be attributed to the yields. Previous solid-phase synthesis of dANP has yielded ~90% yield each cycle[17]. Quickly, in 6 steps, the overall yield goes down to 47%. With the use of template-directed ligation, all substrates are ligated to afford the desired polymer product[21]. Template-directed ligation is also a non-enzymatic reaction. Previously, Xiaoyu Li has determined the reaction conditions for a geometry that is opposite to the one that I worked on, with 5' amine and 3' aldehyde[21]. It will be interesting to discover how the position of the positive charge in my geometry will affect the stability as compared to the previous geometry. The experimental conditions will set a good guideline for the conditions of my geometry.

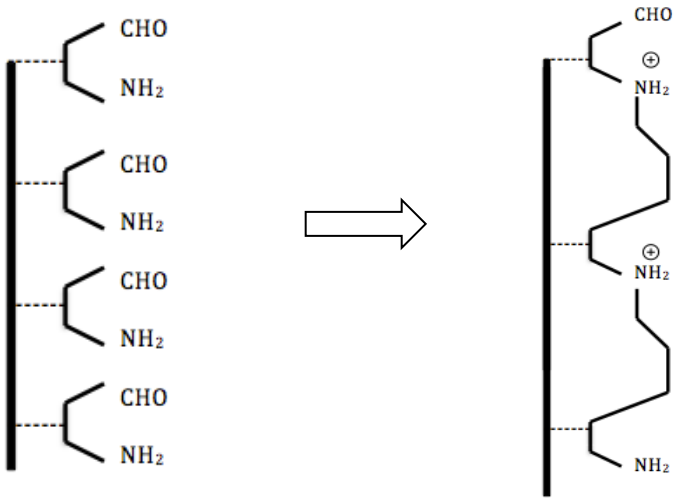


Figure 6. Template-directed ligation of dANP monomers. Close proximity of the functional groups allows imine condensation and subsequent reduction to secondary amien. Thick lines represent dA templates. Dotted lines show base pairing.

Demonstration of cellular uptake and future experiments

To demonstrate cellular uptake of dANPs, BODIPY dye will be used to label the strands at the 5' end. BODIPY dye is neutrally charged, and has been shown to permeate cell membranes of zebrafish embryos[18]. A series of experiments can show entry into the cell. As a control for the experiment, DNA strands with Texas red dye only, will be placed in the presence of Jurkat cells. Since naked DNA strands cannot enter the cell, no signal should be detected inside the cells. BODIPY labeled dANPs will also be placed in the presence of Jurkat cells, separately, to show entry into cell. Subsequently, BODIPY labeled dANPs will be mixed with Texas red labeled DNA to show that the dANP indeed enters the cell and is not adhered to the surface. Detection of two signals within the cells will confirm the expected results. Previous experiments have shown that DNA can be taken up by the cell in the presence of dANPs. (Figure 7)

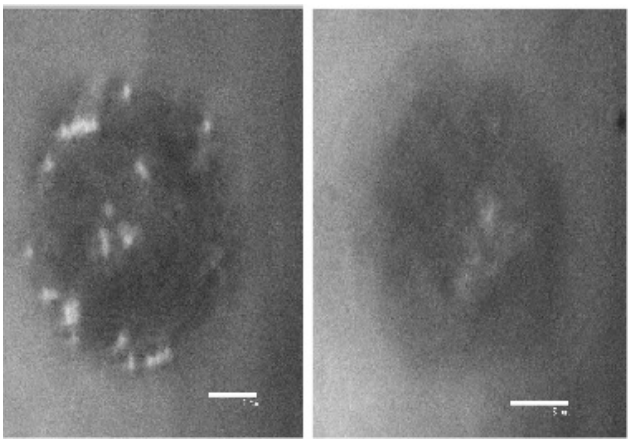


Figure 7. Confocal microscopy image of DNA/dANP cellular uptake. Left: fluorescein labeled dA 8mer/dANP duplex. Right: fluorescein labeled dA 8mer single strand

As to future experiments, the dANPS can be microinjected into zebrafish embryos to observe phenotypic changes. The experiment can also help shed light on toxicity, rate of decay *in vivo*, and areas of aggregation. Currently, only thymidine has been synthesized, but when all other bases are synthesized, many more options will be available. With all bases, a specific sequence from a gene of known function can be obtained from the National Center for Biotechnology Information (NCBI) to test. For example, the complementary sequence of the start of the Pax6a gene can be synthesized. Because Pax6a is vital to the development of the eye and brain structure[19], phenotypic change can be observed with the mixture of dANPs with cells. Upon passively entering the cell, dANPs will hybridize with target mRNAs and shut down protein production at the genetic level. In this way, antisense technology can be very powerful as a regulator without the problems of competition from traditional protein-ligand inhibitory methods.

V. Conclusion

Antisense technology is a promising technique for the treatment of many diseases and infection. When specificity, stability, and delivery are optimized, the antisense oligonucleotides provide for an easy, effective way to turn down gene expression in a very specific and targeted manner. The synthesis of the dANP is not an easy task because of the various protecting groups and stringent reaction conditions. I ran control experiments and started to explore alternatives to increase the yields of the monomer in the final two reactions. Future experiments can be conducted to test reactivity of acetaldehyde ylide to increase yield of monomer for template-directed ligation.

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