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Overcoming degradation: A novel synthetic strategy for antisense oligonucleotide analogs

Annie Lin

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Overcoming Degradation: A Novel Synthetic Strategy for Antisense Oligonucleotide Analogs

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Science and Math
James Madison University

By Annie Lin

May 2019

Accepted by the faculty of the Department of Chemistry and Biochemistry, James Madison University, in partial fulfillment of the requirements for the Honors College.

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Abstract

Antisense oligonucleotides (ASO) are single-stranded deoxyribonucleic acids that bind to mRNA to inhibit the synthesis of proteins that have been associated with the central mechanisms of disease development. Due to their gene silencing capabilities, the potential for ASOs as therapeutic agents is wide, but many toxicological challenges such as poor membrane permeability, low solubility, and rapid degradation by exonucleases must be overcome before ASO medications can be reliably utilized. In order to negate these challenges, the natural sugar-phosphate backbone of ASO's, which is responsible for its rapid degradation, will be replaced by one that is hydrolytically stable. To do so, synthetic oligonucleotide analogues that lack the traditional ribose-phosphate backbone are being developed and will be studied to assess their stability and ability to suppress gene expression.

Introduction

The availability of novel drugs in genetic medicine has the potential to treat neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, and other rare genetic conditions for which there is no current therapy. The majority of approved drugs target the end product of gene translation: the proteins involved in the central mechanisms of disease development. However, as of recent, research has shifted to focus on biologically active molecules that target RNA or DNA as potential therapeutic agents. Among these molecules of interest, a new class of synthetic single stranded molecules of nucleic acids, antisense oligonucleotides (ASOs), have demonstrated promising experimental results due to their ability to target hnRNAs or mRNAs, interfere with mRNA splicing, and arrest gene translation, thus reducing or inhibiting target protein expression.¹

The ability of ASOs to promote a highly specific reduction in the amount of a mRNA makes the clinical application of antisense technologies highly attractive. However, natural ASOs are inherently unstable due to their phosphodiester-sugar backbones, which quickly get degraded *in vivo* via exonucleases into monomers that have deleterious non-specific binding properties.² As a result, modifying the oligonucleotide backbone to increase nuclease resistance has become an important goal in medicinal chemistry research. A variety of modification strategies have been reported, such as altering the phosphodiester moiety (for example, phosphoramidate, methylphosphonate, and phosphorodiamidate morpholino), changing the backbone linkage altogether (as in peptide nucleic acids), and eliminating the target of nucleases, the deoxyribose ribose ring (simplified glycol nucleic acid oligonucleotides).^{3, 4, 5}

Despite containing modified backbones, current ASOs still face many challenges that include, but are not limited to, poor membrane permeability, poor potency, non-specific effects, rapid degradation by exonucleases, and short-shelf life, making them unacceptable as therapeutic drugs.³ To address these issues, a novel synthetic strategy that replaces the traditional ASO sugar backbone with an oligomer derived from seven-membered carbon rings has been developed (Figure 1).

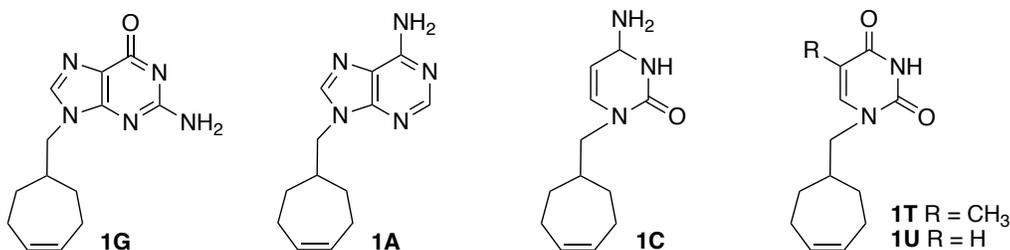


Figure 1. Seven-membered cyclic monomers containing guanine, adenine, cytosine, thymine, and uracil, respectively

Currently, our research aims to synthesize all desirable cyclic monomers. Once the individual bases are made, nuclease-resistant ASOs will be synthesized in one-step from suitable monomers using a DNA templated ring opening metathesis polymerization (TROMP, Figure 2). Following TROMP, heat will be used to break the hydrogen bonds holding the two strands together; the specific product ASO analogue will be extracted and the DNA template will be recycled and reused. Further experiments *in vitro* will be performed to determine and assess the stability, binding specificity, and gene suppression effectiveness of the ASO analog. The aim is to synthesize stable ASO analogs, making the application of oligonucleotides more efficient as systemic drugs.

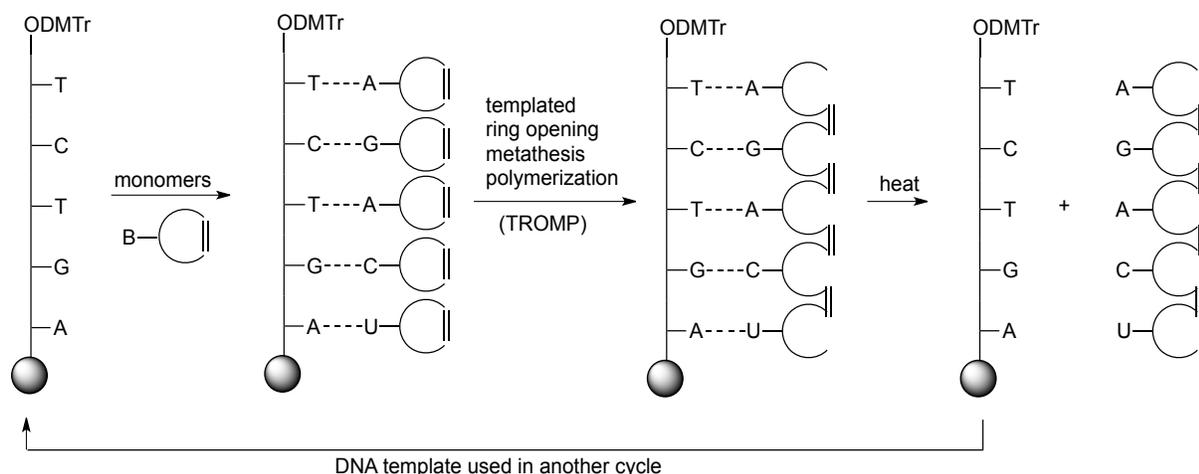
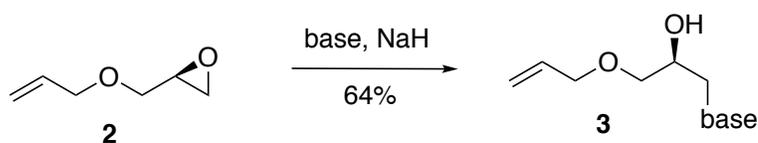


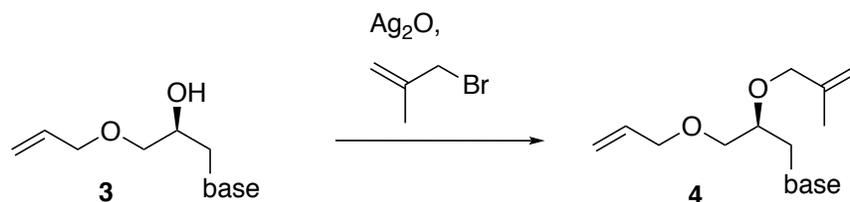
Figure 2. Synthesis of ASO analog via templated ring opening metathesis polymerization

Experimental

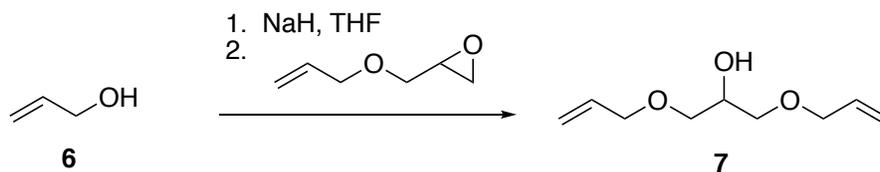
Reactions were carried out under dry N_2 gas. Unless stated otherwise, solvents and reagents were used as obtained from commercial suppliers. Dimethylformamide (DMF), tetrahydrofuran (THF), toluene, and ether are dried and purified via a SPPS system. NMR analyses were conducted on either Bruker Advance DPX-300 or Bruker Advance DRX-400.



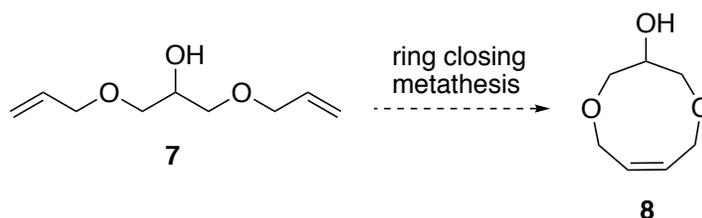
Compound 3T (base = thymine). Sodium hydride (0.305 g, 12.71 mmol), DMF (20 mL), and the base thymine (1.07 equivalent of **2**) were placed in a two-necked flask and stirred under N₂ gas for 2 h. Allyl glycidyl ether **2** (2.30 mL, 28.8 mmol) dissolved in DMF (60 mL) was then added into reaction mixture; the reaction was brought to 110-120 °C and was heated at reflux for 16 h. The DMF was removed via short-path distillation *in vacuo*. The product was extracted from the remaining solid with ethyl acetate, which was then removed using a rotary evaporator. The product was purified via column chromatography (7:3 of hexanes: ethyl acetate); total yield was 64%. ¹H NMR (400 MHz, CDCl₃) δ 7.13 (s, 1H); 5.88 (ddt, *J* = 17.2, 10.4, 5.7 Hz, 1H); 5.28 (ddd, *J* = 17.2, 1.8, 1.5 Hz, 1H); 5.22 (ddd, *J* = 10.4, 1.8, 1.2 Hz, 1H); 4.08 (m, 1H); 4.02 (apparent dt, *J* = 5.7, 1.3 Hz, 2H); 3.97 (dd, *J* = 14.3, 3.2, 1H); 3.69 (dd, *J* = 9.7, 6.0 Hz, 1H); 3.52 (dd, *J* = 9.7, 4.4 Hz, 1H); 3.43 (dd, *J* = 9.7, 6.0 Hz, 1H); 1.89 (s, 1H).



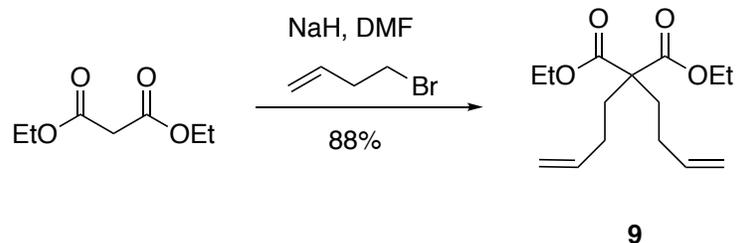
Compound 4T. Compound **3T**, 3-Bromo-2-methylpropene, Ag₂O, and dry DMF were placed in a round-bottomed flask and stirred under N₂ at room temperature for 16 h. The DMF was removed via short-path vacuum distillation, and the crude product was purified via column chromatography (1:1:0.1 of ethyl acetate, hexanes, and triethylamine).



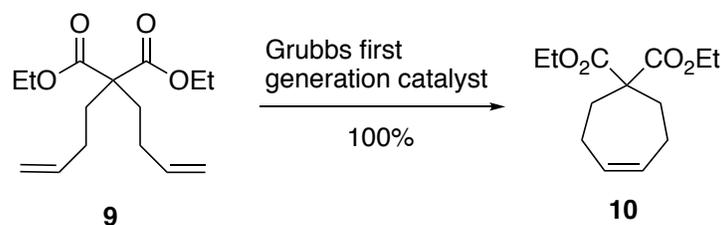
Compound 7. A flask containing NaH (11 g, 275 mmol) and THF (500 mL) was placed in an ice bath; and allyl alcohol (17 mL, 250 mmol) was added dropwise via syringe to the mixture. The ice bath was then removed, and the solution was stirred under N₂ gas for 2 h. Afterwards, allyl glycidyl ether was added to the reaction mixture. The mixture was brought to reflux for 24 h. After cooling to room temperature, methylene chloride (500 mL) was added to the solution. This mixture was washed with water and the organic extracts were dried (MgSO₄), and the solvent was removed via rotary evaporation. The crude product was purified via vacuum fractional distillation (1 mmHg). Four fractions were collected at different temperature ranges: 0-80° C for fraction I, around 80° C for fraction II, 83-115° C for fraction III, and around 115° C for fraction IV. Product (16.573 g, 39%) was collected from fraction III and IV. ¹H NMR (400 MHz, CDCl₃) δ 5.87 (ddt, *J* = 17.4, 10.4, 5.9 Hz, 2H); 5.26 (dd, *J* = 17.6, 1.0, 2H); 5.19 (br d, *J* = 10.4 Hz, 2H); 3.99 (d, *J* = 5.7 Hz, 4H); 3.92 (m, 1H); 3.51 (dd, *J* = 10.7, 4.1, 2H); 3.46 (dd, *J* = 10.7, 6.6 Hz, 2H).



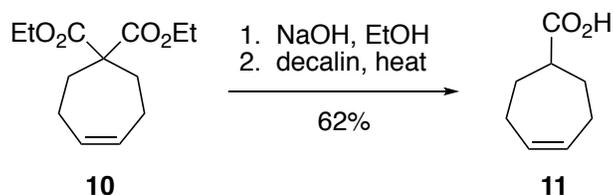
Compound 8. A ring-closing metathesis (RCM) was attempted to convert Compound 7 into **8** under a variety of conditions (**Table 1** in Results and Discussion).



Diethyl-2,2-di-(3-butenyl)malonate (9). Sodium hydride (5.025g, 0.1256 mol) and dry DMF (50 mL) were added into a 250 mL round-bottomed flask, which was placed under N₂, and cooled in an ice bath. Diethylmalonate (16.90 mL, 0.1113 mol) was added dropwise via syringe, and the reaction was stirred at room temperature for 30 min. The mixture was cooled in an ice bath and bromobutene (11.3 mL, 111.3 mmol) was added dropwise via syringe. The mixture continued to stir under N₂ at room temperature for 18 h, after which it was cooled in an ice bath. Another portion of sodium hydride (5.138g, 128.5 mmol) was added in four parts over 45 min. The mixture stirred under N₂ while warming from 0 °C to room temperature for 16 h. The reaction mixture was then stirred for 72 hours. Sodium hydride (2.096g, 84 mmol) was added once more, and the mixture was stirred at room temperature for an hour. Bromobutene (4.40 mL, 40.7 mmol) was also added dropwise to the mixture once again; the reaction was allowed to proceed for 24 h at room temperature. Diethyl ether was then added to the mixture, which was then washed with water 3 times with water. The ether layer was dried over MgSO₄, filtered, and solvent was removed via rotary evaporation, leaving an oil that was filtered through a silica plus to yield 26.372 g (88%) of the desired product. ¹H NMR (400 MHz, CDCl₃) δ 5.80 (ddt, *J*=17.0, 10.3, 7.2 Hz, 2H); 5.03 (ddt, *J*= 17.0, 406, 1.9 Hz, 2H); 5.00 (ddt, *J*= 10.2, 1.9, 1.3 Hz, 2H); 4.20 (quart, *J*, 7.2, 4H); 2.08 (apparent t quart, *J*= 7.2, 1.2 Hz, 4H); 1.90 (m, 4H); 1.26 (t, *J*= 7.1 Hz, 6H).

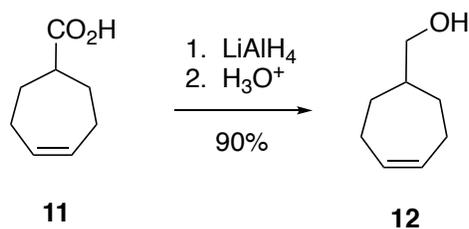


Diethyl 1-cycloheptenedicarboxylate (10). A 2-liter round-bottomed flask containing 0.75 L of degassed dichloromethane, diethyl-2,2-di(3-butenyl)malonate (**9**, 1.015 g, 3.785 mmol), and G1 catalyst (0.150g, 0.182 mmol) was stirred for 5 d. The solvent was then removed, and the crude product was dissolved in hexanes (10 mL) and filtered through silica, which was then washed with 10% ethyl acetate/hexanes. After removal of the solvents via rotary evaporation, a tan oil was obtained (0.906 g, 99.6% yield). ¹H NMR (400 MHz, CDCl₃) δ 5.67 (br s, 2H); 4.21 (quart, *J* = 6.9 Hz, 4H); 2.25 (m, 8H); 1.27 (t, *J* = 7.0 Hz, 6H).

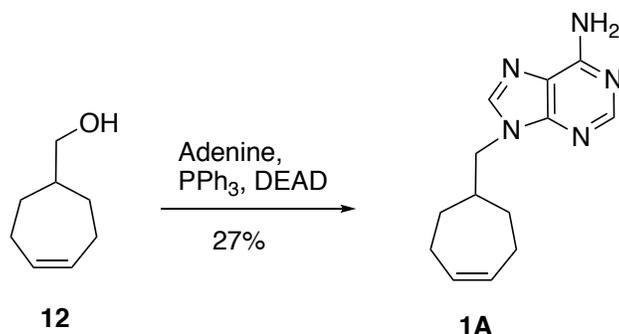


4-Cycloheptene-1-Carboxylic Acid (11). Compound **10** (1.644 g, 6.85 mmol), NaOH (1.436g, 35.9mmol), ethanol (5 mL), water (10 mL), and THF (3 mL) were combined in a round-bottomed flask fitted with a condenser and stir bar. The reaction was brought to reflux under N₂ and kept there for 48 h. Upon cooling to room temperature, water (30 mL) and concentrated HCl were added to the reaction until the pH reached 1-2. The mixture was extracted with ether, dried with MgSO₄, and the solvent removed, leaving a white powder (1.260 g, 100%). A portion of the hydrolyzed product (0.213 g, 1.16 mmol) and DMAP (2.5 g, 21 mmol) were suspended in decalin (7.5 mL) and brought to reflux under N₂ for 28 h. The mixture was cooled and suspended

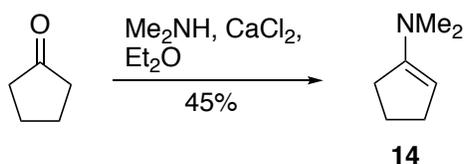
in hexane. A silica column was run, first using hexanes to pull off decalin, then ethyl acetate to elute compound **11** (0.101 g, 62%). ¹H NMR (400 MHz, CDCl₃) δ 5.80 (m, 2H); 2.64 (tt, *J* = 9,7, 4.1 Hz, 1H); 2.33 (m, 2H); 2.13 (m, 2H); 2.03 (m, 2H); 1.69 (m, 2H). Lit:⁷ δ 5.78 (t, 2H, HC=CH), 2.6 (m, 1H, H₂C-CH-CH₂), 2.4 (m, 4H, HC-CH₂-CH₂), 2.2 (m, 4H, HC=CH-CH₂-CH₂).



5-hydroxymethylcycloheptene (12). 4-Cycloheptene-1-carboxylic acid (1.020 g, 6.788 mmol) and diethyl ether (35 mL) were placed in a round bottom flask equipped with a stir bar, condenser, and septum. Lithium aluminum hydride (LAH, 3.0 mL, 7.2 mmol) was added slowly over the course of 45 minutes with the reaction producing rapid evolution of H₂ gas. The flask was swirled intermittently during this addition. Following LAH, the mixture was brought to reflux under N₂ gas for 3 hours then cooled to 0°C. The cold reaction mixture was stirred vigorously and quenched by the dropwise addition of water (6 mL), 15% aqueous sodium chloride (6 mL), and water (2.3 mL). The mixture was filtered and 1/5 of the solids was extracted by THF with a soxhlet apparatus. The THF and filtrate were treated with 7% NaOH (6 mL) and H₂O (2 x 6 mL), dried with MgSO₄ and filtered. The solvent was removed via rotavap to give a pale yellow liquid (0.896 g, 90%). ¹H NMR (400 MHz, CDCl₃) δ 5.78 (t, 2H, HC=CH), 3.47 (d, 2H, CH₂-OH), 2.1/2.08 (m, 4H, HC-CH₂-CH₂), 1.77 (m, 1H, CH₂-CH-CH₂), 1.57 (s, 1H, CH₂-OH).



Compound 1A. 5-hydroxymethylcycloheptene (320 mg, 2.36 mmol) reacted with adenine (401 mg, 2.97 mmol) and triphenylphosphine (PPh₃: 760 mg, 2.89 mmol) in a round-bottomed flask. Under N₂ gas, diethyl azodicarboxylate (DEAD: 0.45 mL, 2.85 mmol) was added dropwise to the reaction mixture via syringe. The reaction was allowed to proceed for 5 d, after which the solvent was removed via rotary evaporation. Dichloromethane (50 mL) was added, and this solution was loaded onto a column (SiO₂ and CH₂Cl₂), from which the product was eluted with 2% MeOH/CH₂Cl₂ (200 mL), followed by 5% MeOH/CH₂Cl₂ (150 mL). Removal of the chromatography solvent gave the desired product (0.170g, 27%). ¹H NMR (300 MHz, CDCl₃) δ 5.8.40 (s, 1H); 7.78 (s, 1H); 5.81 (m, 2H); 5.64 (br s, 2H); 4.09 (d, *J* = 7.4 2H); 1.74 (m, 2H); 1.19 (m, 2H). ¹³C NMR (100 Mhz, CDCl₃) δ 155.4, 153.0, 150.4, 141.0, 134.9, 119.6, 49.9, 42.4, 30.4, 26.3.

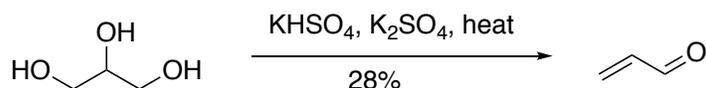


***N,N*-Dimethyl-1-cyclopenten-1-amine (14).** This reaction was done twice, and then combined for the distillation Dimethylamine hydrochloride (40.095 g, 491.66 mmol) was dissolved in a minimum amount of water and added dropwise to a three-necked flask containing

NaOH (40.05g, 1.001 mol). The gaseous product of this addition, dimethylamine, passed through a drying tube containing NaOH before it was condensed and collected in a two-necked flask submerged in a liquid nitrogen/ethyl acetate bath. A slight vacuum was employed to pull off the remaining amine in the reaction flask. The liquid dimethylamine was then added to a round bottom flask, containing CaCl₂ (22.98 g, 207.0 mmol) and dry ether (200 mL). Cyclopentanone (8.0 mL, 90.3 mmol) was also added via syringe. The solution was kept under N₂ at room temperature for 3 d. Afterwards, the solution was filtered and CaCl₂ was washed with dry ether (200 mL).

The second reaction was conducted the same way, using 280.180 g (3.436 mol) of dimethylamine hydrochloride, 280.230 g (7.006 mol) of NaOH, 160.860 g (1.449 mol) of CaCl₂, 56.0 mL (0.632 mol) of cyclopentanone, and 1.0 L of dry ether.

The two reaction mixtures were combined and the ether was removed via rotary evaporation and the remaining solution was distilled under vacuum via vigreux column (bp 80-81 °C at 105 mmHg) to give the enamine (35.860 g, 45%). ¹H NMR (400 MHz, CDCl₃) δ 4.25 (s, 1H, C=CH-CH₂), 2.6 (s, 6H, (CH₃)₂-N), 2.42 (m, 2H, (N-C)-CH₂-CH₂), 2.39 (m, 2H, (C=CH)-CH₂-CH₂), 1.9 (m, 2H, CH₂-CH₂-CH₂).



Acrolein. The apparatus for acrolein synthesis: a 500 mL three-necked flask (**F1**) with pressure equalizing addition funnel, a 100 mL three-necked flask (**F2**), and a second 100 mL three-necked flask (**F3**) submerged in ice (Figure 3). Potassium sulfate (20.001 g, 114.8 mmol),

fused potassium bisulfate (102.58, 753.4 mmol) and 30.000 g of glycerol were added to **F1** and heated to 190-200°C, thus causing the reaction to foam/froth. As **F1** was being heated to 190°C,

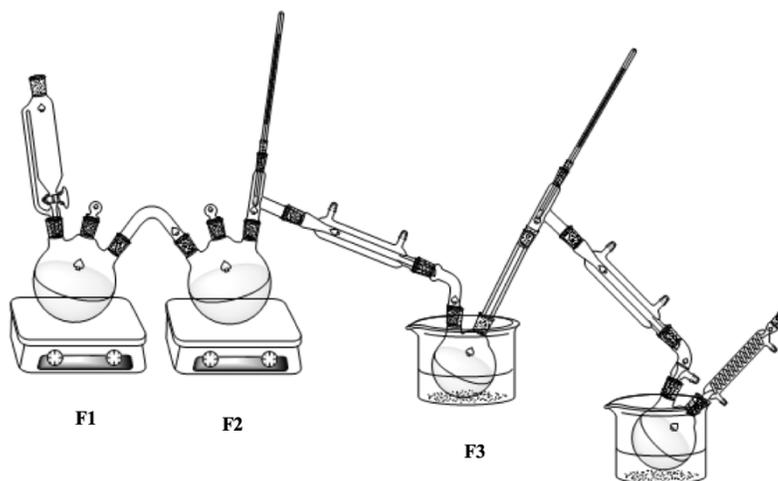
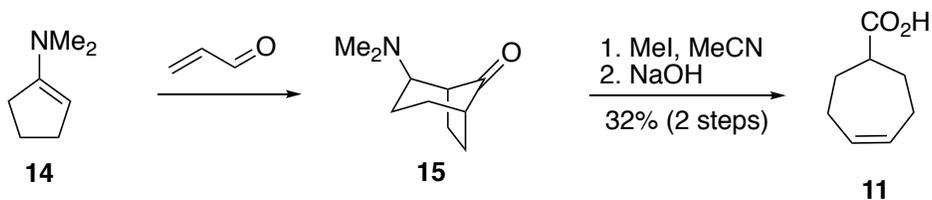
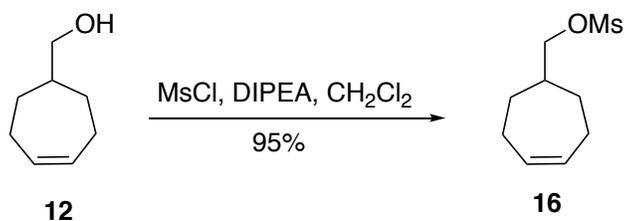


Figure 3. Apparatus setup for acrolein synthesis

F2 was heated to 100-110°C. After the reaction in **F1** proceeded for an h or when the frothing died down, glycerol (30.309 g) was added dropwise to **F1** from the pressure equalizing addition funnel. The reaction in **F1** continued to run for 4 h before the apparatus was disconnected from **F2**. **F2** was then heated for another 1-2 h at 190-200 °C until no more liquid was collecting in F3, which contained about 1 g of hydroquinone and which had been cooled in an ice bath. At this point, **F3** was heated to distill the acrolein into the final flask, which also contained about 1 g of hydroquinone as a stabilizer before being disassembled and storage of acrolein occurred. The product distilled at 53 to 55 °C and was found to weigh 10.202 g (28%). ¹H NMR (400 MHz, CDCl₃) δ 9.55(s, 1H, O=CH), 6.5 (t, 1H, H₂C=CH-(O=CH)), 6.4 (m, 2H, H₂C=CH).

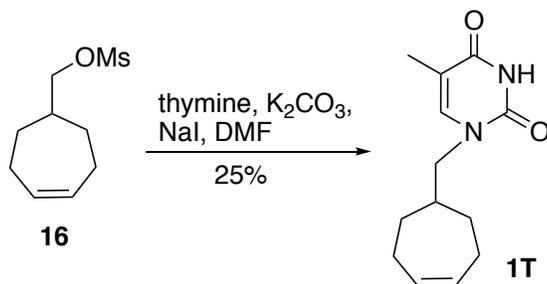


4-Cycloheptene-1-Carboxylic Acid (11). To a round-bottomed flask equipped with a stir bar, placed under nitrogen, and cooled in an ice bath, *N,N*-Dimethyl-1-cyclopenten-1-amine (16.600 g, 149.4 mmol) was added. Following the addition of the enamine, acrolein (10.0 mL, 149.7 mmol) was added dropwise over the course of an h. Upon warming to room temperature, the reaction was stirred for 3 h, then anhydrous acetonitrile (37 mL) was added to the mixture. The reaction was then cooled using an ice bath, and iodomethane (9.5 mL, 152.6 mmol) was added dropwise over the course of 90 min. The resulting solution was then frozen overnight and allowed to thaw out in the morning. After thawing/stirring for 2 h, an aqueous solution of 20% NaOH (147 mL) was added and the resulting mixture was heated to reflux for 19 h. The brown mixture was cooled, and the aqueous layer was extracted with dichloromethane (73 mL). The aqueous layer was acidified with concentrated HCl and a yellow-white precipitate formed. The precipitate was filtered out and the filtrate was again treated with HCl (3 times), allowing more precipitate to form, giving the desired product (7.165g, 32%). ¹H NMR (400 MHz, CDCl₃) δ 5.80 (m, 2H); 2.64 (tt, *J* = 9,7, 4.1 Hz, 1H); 2.33 (m, 2H); 2.13 (m, 2H); 2.03 (m, 2H); 1.69 (m, 2H). Lit:⁷ δ 5.78 (t, 2H, HC=CH), 2.6 (m, 1H, H₂C-CH-CH₂), 2.4 (m, 4H, HC-CH₂-CH₂), 2.2 (m, 4H, HC=CH-CH₂-CH₂).



Mesylate 16. The alcohol **12** (0.105 g, 76.2 mmol) and diisopropylethylamine (DIPEA, 0.216 g, 159 mmol) were added to a 20 mL round-bottomed flask equipped with a stir bar. Dichloromethane (5.0 mL) was added, followed by mesyl chloride (0.061 mL, 78.8 mmol). The flask was capped with a septum and was placed under N₂. The mixture was stirred for 16 h, after

which CH₂Cl₂ (15 mL) was added. This solution was extracted with saturated aqueous NaHCO₃, and the aqueous layer was separated and extracted with CH₂Cl₂ (3 x 10 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo* to give 0.138 g (95%) of the product. ¹H NMR (400 MHz, CDCl₃) δ 5.79 (m, 2H); 4.05 (d, *J* = 6.6 Hz, 2H); 2.30-2.21 (m, 2H); 2.14-2.03 (m, 2H); 2.04-1.95 (m, 2H); 1.87-1.79 (m, 2H); 1.27-1.17 (m, 2H).



Compound 1T. A mixture of mesylate **16** (1.26 g, 3.3 mmol), thymine (462 mg, 3.7 mmol), dried K₂CO₃ (1.1 g, 8.0 mmol), and NaI (600 mg, 4.0 mmol) in dry DMF (50 mL) was stirred at 90°C for 16 h. The reaction mixture was cooled and evaporated; the residue was dissolved in ethyl acetate (50 mL). The organic layer was washed with saturated NaHCO₃ solution (25 mL) and H₂O (2 x 20 mL), dried, and evaporated. The crude product was then purified by column chromatography, affording 25% yield. ¹H NMR (400 MHz, CDCl₃) δ 6.94 (s, 1H); 5.79 (m, 2H); 3.55 (d, *J* = 7.5 Hz, 2H); 2.30-2.17 (m, 2H); 2.13-1.99 (m, 3H); 1.78-1.65 (m, 2H); 1.20-1.06 (m, 2H).

Results and Discussion

The initial monomers: design and attempted synthesis

The initial approach involved the synthesis of the monomers shown in Figure 4. These compounds were chosen because each included an eight-membered ring containing a double bond, a ring size that is known to be effective in ring-opening metathesis polymerization. We

planned to prepare monomers containing five DNA/RNA bases from a chiral allyl glycidol ether via the same general strategy (Scheme 1).

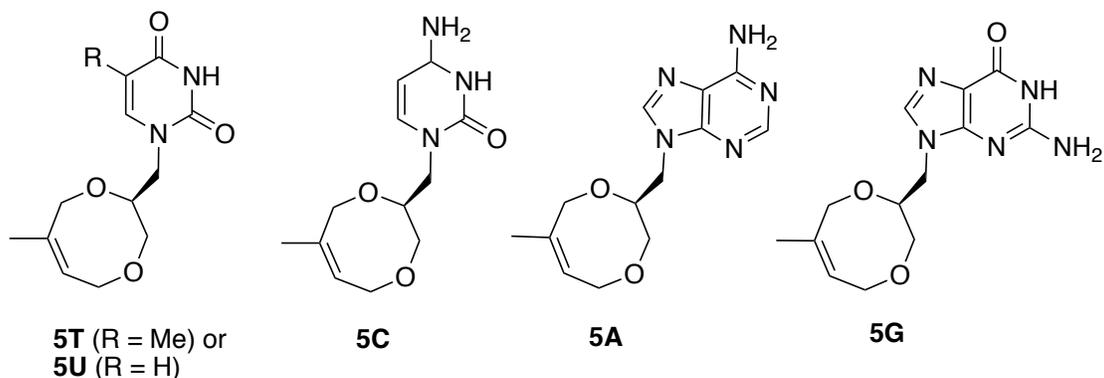
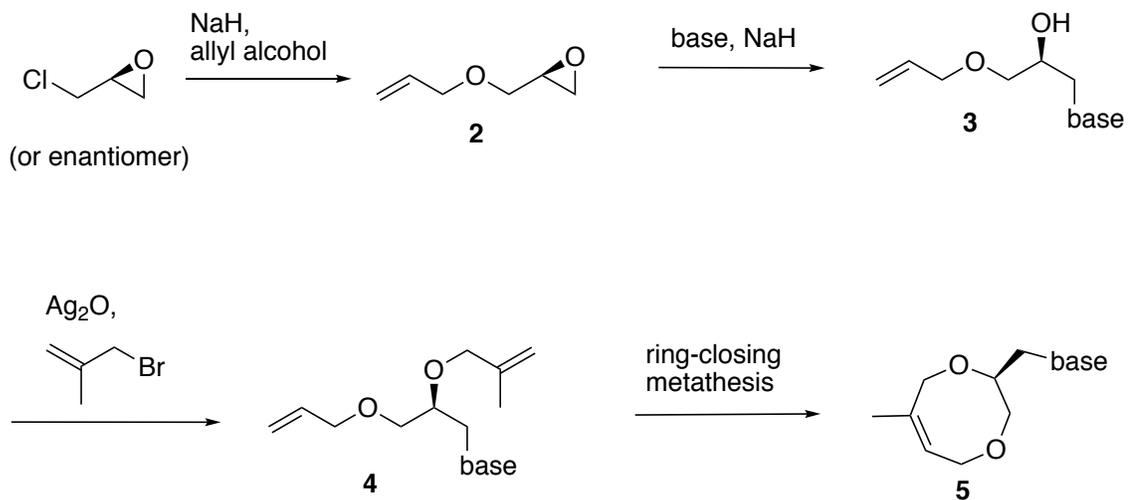


Figure 4. Cyclic monomers from Scheme 1

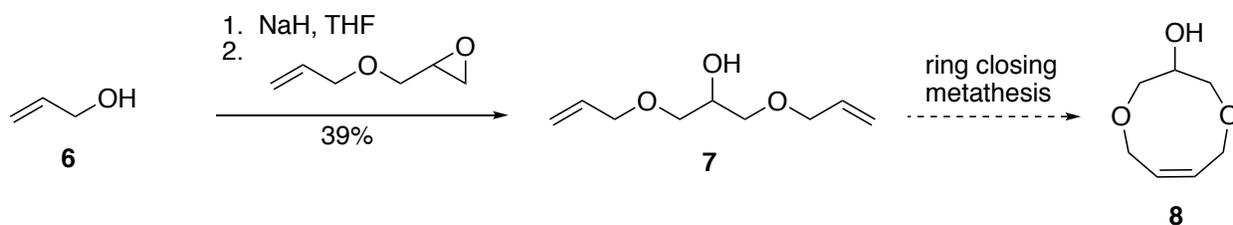
The exocyclic amino groups of guanine and adenine would be protected as *N*-dimethylformamides, and the amino group of cytosine would be protected via an acetamide. Because no protection is needed for thymine, the synthesis of **5T** was attempted first. Allyl glycidyl ether (**2**) was initially used as a mixture of enantiomers from a commercial source. If this entire synthetic sequence worked, each enantiomer of **2** and subsequent compounds would have been produced from the individual enantiomers of compound **1**.



Scheme 1

In the first step, a racemic mixture of ally glycidyl ether **2** was employed for the synthesis of compound **3T** (base = thymine); the purified product was produced in 75% yield and was confirmed to be **3T** via ^1H NMR spectroscopy. In the second step, 3-bromo-2-methylpropene and Ag_2O were used to convert compound **3T** to **4T**, however the attempted synthesis was unsuccessful. The desired product may have been lost or stuck to the silica used during column chromatography. The synthesis in Scheme 1 was found to be difficult over time due to problems with the conversion of **3T** to **4T**. In addition, the overall strategy would be inefficient and time-consuming because all five bases were installed in the initial step, causing five different parallel pathways to be necessary obtain the five equivalent cyclic monomers; therefore, the approach shown in scheme 2 was developed to overcome this limitation, with all monomers prepared from the same alcohol **8**.

The second, more efficient approach



Scheme 2

The monomers chosen included a nine-membered ring containing two oxygens and a double bond (Figure 5). The strategy was to first synthesize the precursor **8**, a nine-membered ring substituted with a hydroxymethyl group, via one common pathway for all monomers, then via a subsequent reaction, the -OH group would be replaced with any of the five nucleobases, yielding the desired cyclic monomer.

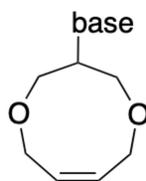


Figure 5. Nine-membered ring monomers to be prepared from **8**

Thus, to synthesize the cyclic monomers, allyl alcohol reacted with allyl glycidyl ether (**2**) under basic conditions in THF, to yield the symmetrical alcohol **3** (38.5% yield). The product structure was confirmed by ^1H NMR and mass spectroscopic analysis. Compound **3**, under condition that promote ring-closing metathesis (RCM), was converted into a nine-membered ring containing two oxygens. Trials involving a slight variation of ring-closing conditions, summarized in **Table 1**, were conducted to identify the most efficient and high-yielding synthetic method.

Table 1. Conditions for ring-closing metathesis.

Trial	Catalyst* (amount)	Substrate molarity	Solvent	Additive (M)	Temp. (°C)	Time
1	G1 (10 mol %)	0.01	toluene	Ti(O- <i>i</i> pr) ₄ (0.02)	25	96 h
2	G2 (10 mol %)	0.01	toluene	Ti(O- <i>i</i> pr) ₄ (0.02)	25	48 h
3	G2 (10 mol %)	0.01	toluene	—	25	48 h
4	G2 (10 mol %)	0.01	toluene	Ti(O- <i>i</i> pr) ₄ (0.02)	110	40 min
5	G1 (10 mol %)	0.01	toluene	—	25	96 h
6	G2 (10 mol %) [†]	0.1	toluene	—	110	40 min
7	G1 (10 mol %) [#]	0.01	toluene	Ti(O- <i>i</i> pr) ₄ (0.02)	110	24 h
8	G1 (20 mol %) [†]	0.0012	CH ₂ Cl ₂	—	40	48 h
9	G1 (20 mol %) [†]	0.0005	CH ₂ Cl ₂	—	40	72 h
10	G1 (20 mol %)	0.00058	toluene	—	110	40 min
11	G1 (20 mol %)	0.00029	toluene	Benzoquinone (0.00058)	110	48 h

*G1 and G2 are Grubbs first- and second-generation catalysts, respectively.

[#]G1 catalyst was added after 24 h reflux of substrate with Ti(O-*i*pr)₄; mixture was heated at reflux for 40 additional minutes.

[†]After 24 hours, a second portion of catalyst, in the same amount, was added.

After multiple trials and attempts to promote ring-closing, the desired product was obtained but this approach was not practical due to difficulty and complexity associated with the synthesis of a nine-membered ring, which was identified by previous studies in the chemical literature as one of the hardest ring sizes to synthesize.⁶ Therefore, the approach shown in Scheme 3 was developed in an attempt to utilize a smaller ring that is more readily formed.

The third synthetic approach: utilizing a 7-membered ring

The monomers (Figure 6) chosen included a seven-membered ring containing a double bond, with the initial route given in Scheme 3.

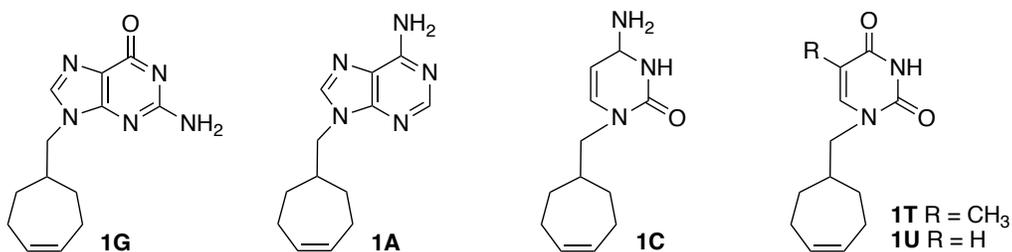
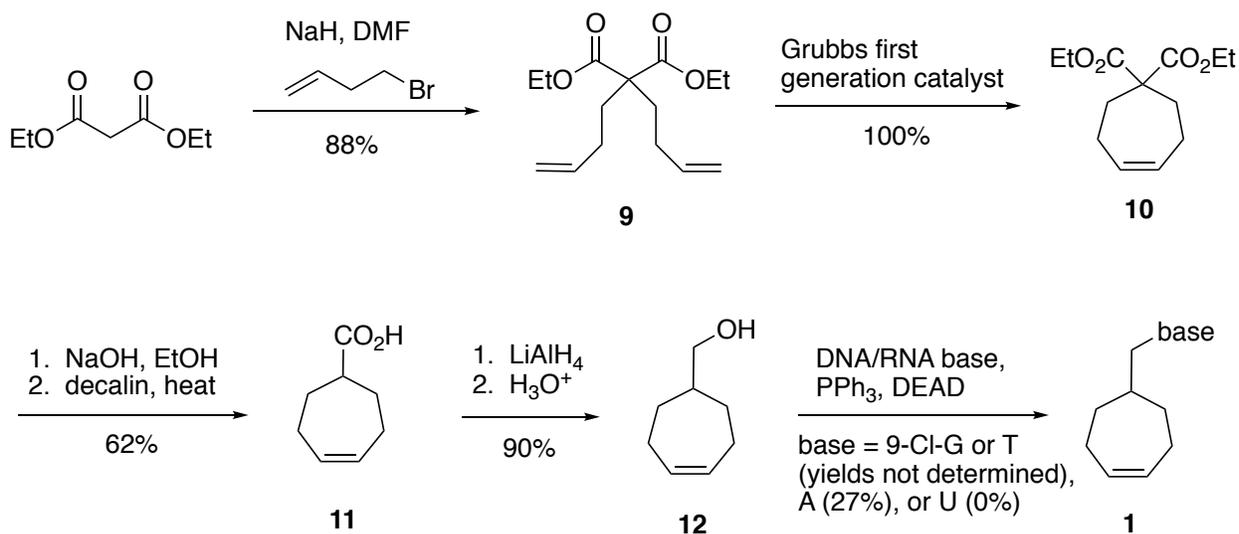


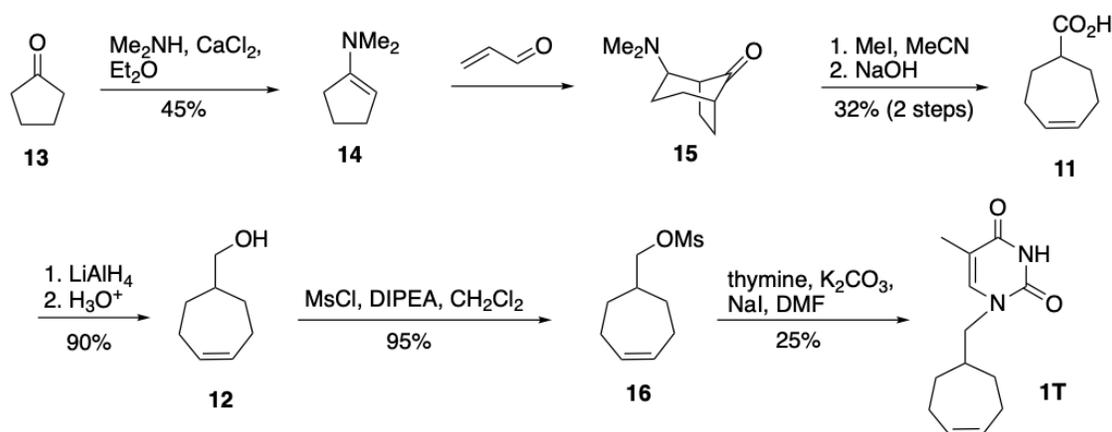
Figure 6. Seven-membered ring monomers from Scheme 2



Scheme 3

This route began with the production diethyl-2,2-di-(3-butenyl)malonate from a reaction of diethylmalonate, sodium hydride, and 4-bromo-1-butene. The successful production of diethyl-2,2-di-(3-butenyl)malonate was confirmed by ^1H NMR spectroscopy; the yield for this step was 88%. The diethyl-2,2-di-(3-butenyl)malonate then proceeded to undergo a ring closing metathesis quantitatively and hydrolysis/decarboxylation to generate 4-cycloheptene-1-carboxylic acid in 62% yield. The carboxylic acid was then reduced into 5-hydroxymethylcycloheptene by LAH in a yield of 90%. Subjecting 5-hydroxymethylcycloheptene (**12**) to Mitsunobu reactions employing adenine, uracil, thymine, and a precursor to guanine to produce **1A**, **1U**, **1T**, or a precursor to **1G** gave mixed results. The adenine, thymine, and guanine precursor analogs were successfully produced, but the reaction with uracil did not work.

All compounds were characterized via ^1H NMR spectroscopy. Due to limitations in the amount of material that could undergo the ring-closing metathesis at one time, the strategy Scheme 3 was modified to increase the production of 5-hydroxymethylcycloheptene, which is the precursor to the cyclic monomers (Scheme 4).



Scheme 4

The route in Scheme 4 employs a known synthesis of the desired 5-hydroxymethylcycloheptene (**12**), the precursor to the addition of the nucleobases. This preparation involved adapting the procedure of Marquardt/Newcomb and Adkins/Hartung^{7,8} In addition, the Mitsunobu reaction was abandoned in favor of a nucleophilic substitution to attach the nucleobases.

Thus, we began with the synthesis of *N,N*-Dimethyl-1-cyclopenten-1-amine (45%) from a reaction between dimethylamine hydrochloride and sodium hydroxide, followed by cyclopentanone. This compound was purified via vacuum distillation. The presence of the enamine was confirmed by ¹H NMR analysis. The signal at 4.25 ppm indicated the presence of the double bond in the cyclopentene, and the signal at 2.6 ppm with an integration of 6 showed the presence of the methyl groups attached to a nitrogen.

N,N-dimethyl-1-cyclopenten-1-amine then reacted with acrolein, iodomethane, and a 20% aqueous solution of sodium hydroxide to produce 4-cycloheptene-1-carboxylic acid (32%). Since acrolein is not commercially available in small quantities, it was synthesized in lab from glycerol, potassium sulfate, and potassium bisulfate with a yield of 28%. The structures of acrolein and 4-cycloheptene-1-carboxylic acid were confirmed via ¹H NMR spectroscopy. The aldehyde hydrogen of the acrolein corresponded to the peak at 9.5 ppm, and 4-cycloheptene-1-carboxylic acid was confirmed by the presence of signals at 5.78, 2.01, 1.99, 1.98/97, and 1.72 ppm.

The 4-cycloheptene-1-carboxylic acid was then reduced with lithium aluminium hydride to produce 5-hydroxymethylcycloheptene in 90% yield. The product was confirmed via ¹H NMR, which showed peaks at 5.78, 3.47, and 1.57 ppm, indicating the presence of the double

bond on the ring, the methylene group coming off the ring, and the hydrogen in the alcohol group, respectively.

Currently, we are preparing all five of the analogs of **5** via the production of the mesylate **16**, which is then subjected to a nucleophilic substitution reaction to attach the nucleobases. Thus, treatment of the alcohol **12** with mesyl chloride and an amine base in dichloromethane has provided excellent yield of the mesylate **16**.⁹ This compound has been successfully converted into **1T**, **1U**, and **1A** under a variety of conditions.¹⁰ We expect to have **1G** and **1C** in hand soon using this same strategy.

Conclusion

The main goal of this research is to streamline the synthesis of antisense oligonucleotide analogues, ultimately making the application of oligonucleotides more effective and efficient as systemic drugs. To do so, a novel strategy of replacing the traditional ASO backbone with a carbon ring backbone was attempted in four synthetic schemes. Each approach aimed to create a cyclic ring that can be attached to each nucleobase, forming cyclic monomers that can be polymerized into ASO analogs.

The first ring system attempted (Scheme 1) may have ultimately been successful; but it was abandoned because it required each analog to be synthesized via a separate route. Therefore, we sought an approach in which the nucleobases would be attached in the last step to a common intermediate, (**8**, Scheme 2). Unfortunately, the ring-closing metathesis of **7** to form **8** proved to be difficult (Table 1), so a completely new ring system was chosen (Figure 6). The initial synthesis of these compounds also employed a common intermediate (**12**, Scheme 3), and it successfully produced two analogs (**5A** and **5-Cl-G**, a precursor to **5G**). Although this route employed a common intermediate and a high-yielding ring closure, the latter reaction had to be

done at extraordinarily low concentration, requiring impractical amounts of solvent. In addition, the Mitsunobu reaction done in the last step produced some PPh₃-DEAD derived side product that co-purified with the product in some instances. As a result, the approach in Scheme 4 was developed. This strategy made use of an old literature preparation of **11**, and employed an S_N2 reaction in place of the Mitsunobu to attach the nucleobases.

Once all nucleotides have been synthesized, ring opening metathesis polymerization reactions will be performed to synthesize full length ASO strands. Initially, we will study each monomer's conversion to a 20-mer of oligo-5* via a polymerization templated on a 20-mer of its complementary oligomer in solution and on a bead (both of which can be obtained commercially). Future work includes assessing the strength and specificity of binding of the modified oligomers to target sequences of RNA and DNA via melting temperature curves.¹¹ If they do bind with specificity for the target sequences, an RNaseH resistance assay will be conducted. Longer term goals will involve studying our modified oligonucleotides' ability to silence or suppress gene expression in *E. coli* cells that have been engineered to express the reporter protein beta-galactosidase.

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