Structural Analysis of Detrimental Oxidative Products from Phenolic Carcinogens and Construction of Nucleotide Tools from Analogous Processes.

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ABSTRACT

Structural Analysis of Detrimental Oxidation Products from Phenolic Carcinogens and Construction of Nucleotide Tools from Analogous Oxidative Processes.

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Toxicological research involves multiple cellular targets, but perhaps the most prominent is DNA. The importance of this molecule for life, and the many phenotypical consequences from its modification, necessitates the study of toxicant interaction with DNA. Phenols are ubiquitous in nature, possess positive and negative cellular effects, and are primary metabolites of several established carcinogens. Phenol toxicity is oxidatively mediated and oxidation of phenols can produce phenoxy radicals that react directly at the C8 position of guanine to afford both carbon-linked and oxygen-linked C8-2'-deoxyguanosine (dG) adducts. Phenols also undergo oxidation to form catechols, which redox cycle to produce quinones. This process exacerbates oxidative stress in cells and promotes covalent attachment to cellular macromolecules, as quinones are susceptible to nucleophilic attack.

O-linked-C8-guanine adducts were studied in DNA, expanding upon previous work in the Manderville laboratory on the simplest O-linked adduct, C8-phenoxy-2'-deoxyguanosine (PhOdG). The impact of phenyl ring expansion and chlorination of the PhOdG adduct in a duplex environment was studied, and suggested that pi-stacking and lipophilicity affect adduct orientation within the helix, which may rationalize mutagenic
outcomes in human DNA. Mass spectrometry was used to elucidate fragmentation pathways for effective dose biomarker detection from exposure to chlorinated phenols.

Oxidative reactivity of the phenolic C-linked adduct (p-PhOH-dG) was also assessed, as this adduct possesses a lowered oxidation potential, and is susceptible to targeted oxidative attack to generate secondary oxidation products. Hydroxyl radical addition to p-PhOH-dG formed a catechol, which subsequently oxidized to a quinone adduct, and was trapped by a sulphur nucleophile. This previously unexplored oxidative pathway may contribute to phenol toxicity.

Formation of reactive centers from oxidative processes on guanine adducts encouraged their use to confer covalent reactivity to functional oligonucleotides (aptamers). Furan modified aptamers for thrombin were synthesized and site selectively reacted with nucleophiles to afford covalent products. Linking aptamers to their targets allows for identification of unknown protein targets, and enhances aptamer therapeutic effects.

The body of work herein provides mechanistic toxicological information for phenol adducts in DNA and uses oxidative reactivity of C8 guanine adducts to functionalize aptamers for utility in industrial applications.
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Figure 3 - 14  ESI−MS analysis of product from the aqueous NH4OH (30%) decomposition of 3″,4″-DHPH-dG. (A) ESI−MS spectrum with [M − H]− = 406. (B) MS3 spectrum taken at m/z 290.
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Figure 3 - 16  ESI⁺-MS analysis of proposed diacid product from the aqueous NH₄OH (30%) decomposition of 3″,4″-DHPh-dG. (A) ESI⁺-MS spectrum with [M + H]⁺ = 408 and major fragment at m/z 292 for loss of deoxyribose (-116). (B) MS³ spectrum taken at m/z 292, (C) MS⁴ spectrum taken at m/z 275, (D) MS⁵ spectrum taken at m/z 258, and (E) MS⁶ spectrum taken at m/z 230.

Figure 3 - 17  HPLC elution profile of the aqueous NH₄OH (10%) decomposition of 3″,4″-DHPh-dG in the presence of 50 equiv NAC: (i) initial 3″,4″-DHPh-dG and following treatment for (ii) 30 min, (iii) 4 h, and (iv) 24 h at ambient temperature.

Figure 3 - 18  ESI⁻-MS spectrum of peak labeled (*) for the mono-NAC conjugate (NAC-3″,4″-DPh-dG), MS³ taken at m/z 406 is shown in the insert.

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Figure 4 - 1  SELEX selection process for screening aptamer libraries.

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Figure 4 - 3  A) G-tetrad in TBA stabilized by metal ion. B) Crystal structure of TBA. C) C8-aryl-guanine modification sites in TBA at G₅, G₆ and G₈. Reproduced with permission from reference 45.
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Figure 4 - 5  Ring opening of furan to form cis-butene-1,4-dial and subsequent conjugation with glutathione (GSH) and lysine to form double conjugated pyrrole adducts.

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Figure 4 - 7  A) HPLC trace showing (1) pre-oxidation with NBS. B) HPLC trace of (1) after addition of 5 equiv of NBS over 40 mins. C) ESI⁺-MS for product peak i) from oxidation of (1). D) ESI⁺-MS for product peak ii) from oxidation of (1).

Figure 4 - 8  A) UV-vis absorbance spectra of oxidation product peak (i) from (1). B) UV-vis absorbance spectrum of oxidation product peak (ii) from (1).

Figure 4 - 9  A) HPLC trace of (2) before oxidation. B) HPLC trace of (2) after addition of 4 equiv NBS over 30 min. C) ESI⁺-MS for oxidation peak i). D) UV-vis absorbance spectrum of oxidation peak i).

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Figure 4 - 11  A) ESI⁻-MS for peak bi) in Figure 11B representing product (4) which is the NAC trapped adduct of ring opened species from oxidation of (2) with NBS. B) UV-vis absorbance spectrum of peak bi).

Figure 4 - 12  ESI⁻-MS for isolated peak c) from the addition of 20 equiv NAL to the reaction mixture after stirring for 2 h at 37 °C.

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Figure 4 - 18  HPLC showing oxidation and trapping studies using 3fur-TBA modified at G5. A) Starting strand. B) 4 equiv of NBS added over 30 min. C) NAC NAL added simultaneously with stirring at 37 °C for 14 h. D) Treatment with 10% formic acid at 70 °C for 1 h. E) NAC-NAL-3fur-G (6) standard.

Figure 4 - 19  PAGE gel stained with Coomassie blue showing modified TBA oxidation cross-linking reactions. Well designations: 1) Protein ladder, 2) BSA, 3) Bovine thrombin protein 4-6) 3-furyl mTBA at G8 7-9) 3-furyl mTBA at G6.

Figure 4 - 20  TBA-thrombin binding complex. Reproduced from crystallography file HAL1 showing G6 in yellow.

Figure B - 1  MS Spectrum for NaphO G modified Narl 12mer sequence at the G3 position.

Figure B - 2  MS Spectrum for PPhO G modified Narl 12mer sequence at the G3 position.

Figure B - 3  MS Spectrum for CNBPO G modified Narl 12mer sequence at the G3 position.

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Figure B - 5  Oligonucleotide fragment-ion nomenclature proposed by McLuckey.

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Figure B - 7  MS Spectrum for PCPO G modified Narl 12mer sequence at the G3 position.

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Figure B - 11  ESI$^-$-MS$^3$ Spectrum for $^{13}$NarG modified Narl 12mer sequence at the G$_3$ position.

Figure B - 12  MS Spectrum for 3"-4"-DPh-dG.

Figure B - 13  MS Spectrum for 3fur-G modified TBA at position G$_5$.

Figure B - 14  MS Spectrum for 3fur-G modified TBA at position G$_6$.

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LIST OF ABBREVIATIONS

2fur-dG 8-(2-furyl)-2'-deoxyguanosine
3"',4"'-DHPh-dG 8-(3"',4"'-dihydroxyphenyl)-2'-deoxyguanosine
3fur-dG 8-(3-furyl)-2'-deoxyguanosine
8-Br-dG 8-bromo-2'-deoxyguanosine
8-oxo-dG 7,8-dihydro-8-oxo-2'-deoxyguanosine
A adenine
A.U. arbitrary unit
AAF 2-(acetylamino)fluorene
AAF-dG 8-(2-acetylaminofluorene)-2'-deoxyguanosine
ACN acetonitrile
AF aminofluorene
AFB1 aflatoxin B1
AF-dG 8-aminofluorene-2'-deoxyguanosine
AN-dG 8-aminophenyl-2'-deoxyguanosine
ABP-dG 8-(aminobiphenyl)-2'-deoxyguanosine
aq. aqueous
B major groove binding conformation - B-type
BER base excision repair enzyme
BP benzo(a)pyrene
bs broad singlet
Bn benzyl group
C cytosine
calcd. calculated
<table>
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<th>Abbreviation</th>
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<td>CD</td>
<td>circular dichroism</td>
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<td>cytochrome P450 enzyme</td>
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<td>d</td>
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<tr>
<td>ETC</td>
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</tbody>
</table>
MALDI  matrix assisted laser desorption/ionization
MD    molecular dynamic simulations
mer   denotes length of an oligonucleotide
min   minute(s)
MOPS  3-(N-morpholino)propanesulfonic acid
mRNA  messenger ribonucleic acid
MS    mass spectrometry
mTBA  modified thrombin binding aptamer
N.H.E. normal hydrogen electrode
NAC   N-acetylcysteine
NAL   Nα-acetyl-L-lysine
NaphOdg 8-napthoxy-2'-deoxyguanosine
NarI  5'-CTCGGCGCCATC-3'
NarI' (N = −2) 5'-GATGGCCGAG-3'
NarI'(N = C) 5'-GATGGCGCCGAG-3'
NarI'(N = G) 5'-GATGGGGCCGAG-3'
NarI'(N = THF) 5'-GATGG-THF-GCCGAG-3'
NBS   N-bromosuccinimide
NMR   nuclear magnetic resonance
OAc   acetate
OTA   ochratoxin A
p-PhOH 8-(4''-hydroxyphenyl)-2'-deoxyguanosine
PAH   polyaromatic hydrocarbon
PCB   polychlorinated biphenyl
PCP   pentachlorophenol
PCPO$_{dG}$ 8-(2,3,4,5,6-pentachlorophenoxy)-2'-deoxyguanosine
PhO$_{dG}$ 8-phenoxy-2'-deoxyguanosine
PhPhO$_{dG}$ 8-phenylphenoxy-2'-phenoxy pyridine
POP persistent organic pollutant
ppm parts per million
RNA ribonucleic acid
ROS reactive oxygen species
s seconds or singlet
S base displaced stacked conformation - stacked
Sp spiroiminodihydantoin
S$_{n}$Ar nucleophilic aromatic substitution
T thymine or temperature
$t_{1/2}$ half-life
TBA thrombin binding aptamer
TBAF tetrabutylammonium fluoride
TBS-Cl $tert$-butyldimethylsilyl chloride
TCP trichlorophenol
TCP$_{dG}$ 8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine
TEA triethylamine
TEAA triethylammonium acetate
THF tetrahydrofuran
$T_{m}$ thermal melting
TMSEtOH trimethylsilyl ethanol
TPPTS 3,3',3''-Phosphanetriyltris(benzenesulfonic acid) trisodium salt
Tyr tyrosine
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV-vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>W</td>
<td>minor groove binding conformation - wedge</td>
</tr>
<tr>
<td>W-C</td>
<td>Watson and Crick</td>
</tr>
<tr>
<td>ϕ</td>
<td>Fluorescent quantum yield</td>
</tr>
<tr>
<td>Θ</td>
<td>molar ellipticity</td>
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<td>maximum fluorescence excitation wavelength</td>
</tr>
<tr>
<td>λ_{em} (max)</td>
<td>Maximum fluorescence emission wavelength</td>
</tr>
</tbody>
</table>
Chapter 1.
Introduction
1.1 Background Information and Literature Review

1.1.1. DNA General Information

The biopolymer deoxyribonucleic acid (DNA) is, arguably, life’s most important molecule and is essential for the survival and evolution of all eukaryotic species on the planet.\(^1\)\(^-\)\(^2\) The specialized non-covalent interactions possessed by this molecule allow for construction of a highly conserved genetic code responsible for proper cellular growth and reproduction.\(^1\) The stability of information transmission between generations, required for proper functioning of a complex organism, is only possible if replication occurs with high fidelity.\(^2\) Proper maintenance of DNA to conserve these important interactions is, therefore, a major energetic focus of the cell and a focal point in disease prevention and therapy.\(^3\),\(^4\)

![Figure 1](image)

**Figure 1 - 1:** The four different nucleoside units that are used to construct DNA.

DNA itself is a polymer composed of four different nitrogenous bases bound by a glycosidic bond to the anomic carbon of a 2′-deoxyribose sugar.\(^1\) The sugar base unit is called a nucleoside. Nucleosides are connected by a phosphate group that forms a diester bond between the 3′-site on one sugar and the 5′-site on the adjacent sugar molecule, to afford a polymeric chain.\(^1\) The nucleosides themselves are shown in Figure
1-1 and can be divided into two categories: the purines, which include deoxyadenosine (dA) (1), deoxyguanosine (dG) (2); and the pyrimidines, comprised of deoxycytidine (dC) (3) and deoxythymidine (dT) (4).1

Figure 1 - 2: B-form DNA double helix showing the right-handed turn and the major and minor grooves.

Interactions between monomers in a specific and conserved fashion forms a double stranded structure referred to as a DNA helix (Figure 1-2). To form this structure, two strands come together, aligned antiparallel and complementary to each other. Hydrogen bonding interactions between complementary bases, stacking interactions between adjacent bases in the interior of the helix, and polar interactions between the charged phosphate groups on the exterior of the helix, all contribute to the formation and stability of the well known double helical structure (Figure 1-2).1
This structure, first proposed in 1953, was published in *Nature* using the combined efforts of Watson and Crick and the X-ray crystallographic images obtained by Rosalin Franklin.\(^5\) Hydrogen bonding interactions between polymer chains are specific in normal DNA and involve two bonds between adenine and thymine and three bonds between guanine and cytosine (Figure 1-3). The hydrophilic backbone, composed of charged phosphate molecules and deoxyribose sugars, resides on the outside of the helix, facing the aqueous bulk solvent of the cell. The hydrophobic nucleobases reside in the interior of the helix, which possesses a dielectric constant (\(\epsilon\)) of 3-5.\(^6\) Planarity of the nucleobase ring systems allow \(\pi\)-stacking, which stabilizes the secondary helical structure.

The two strands paired together wind around a common axis to form a right-handed helix, which is slightly offset, facilitating a major groove and minor groove on the surface of the helix (Figure 1-2). DNA paired with proper W-C interactions under normal physiological conditions is known as B-form DNA.\(^7\) Variations to normal B-form DNA have been found and are referred to as A-form and Z-form.\(^8\) A-form is commonly observed under dehydrated conditions, and, although it maintains the right-handed helical rotation, it is shorter and more compact with a deeper major groove and more shallow minor groove.\(^7\) Z-DNA has a left-handed helical axis and is much less commonly encountered. This DNA conformation usually arises with stretches of guanines and cytosines and is thought to play a role in gene regulation.\(^9\)
Figure 1 - 3: Normal Watson and Crick (W-C) base pairing of anti-parallel complementary strands of DNA. G-C contains three H-bonds and A-T contains two H-bonds.

Long pieces of DNA duplexes containing ~ 10-100 monomer units are referred to as oligonucleotides. A grouping of thousands of monomer units make up our chromosomes and sections of these long DNA pieces, called genes, serve as the instruction manual for production of all cellular proteins. The process of taking these instructions from where they reside in the nucleus and producing viable protein products in the cytosol of cells is carried out by multiple enzymes. DNA replication in the nucleus is accomplished by DNA polymerase enzymes. The conversion of DNA sequences into
a single stranded mRNA transcripts, which are refined into an efficient signal and transferred to the cytoplasm, is the role of RNA polymerase and transcription factors.\textsuperscript{11,12} Creation of a polypeptide chain by ribosomes, which use the mRNA strand as a template and build amino acid chains sequentially, is the final step in the process.\textsuperscript{1,13} All of these reactions rely on a combination of precise signalling events, presence of necessary enzymatic machinery, and availability of unmodified, abundant substrates. Therefore, despite the overwhelming importance of correct DNA sequences, there are many pitfalls for successful transmission of the genetic information carried within.\textsuperscript{2,13}

1.1.2. Alternative DNA Structures

Although most of the genetic material follows the normal B-form structural template, perturbations have been reported that serve various biological functions or are a result of differing chemical conditions.\textsuperscript{14} For example, A-T rich regions can exist as stable unwound segments and act as origins for DNA replication.\textsuperscript{15} G-T rich sequences, found at the end of chromosomes, can fold into differing secondary structures like triplexes and quadruplexes and serve protective roles, maintaining the integrity of the chromosomes as they are replicated.\textsuperscript{15} Other alternate structures, including cruciforms, are formed by intrastrand interactions between a single strand of DNA.\textsuperscript{15}

\textbf{Figure 1 - 4: The normal W-C binding face of a nucleoside and the alternate Hoogsteen binding face used for alternative DNA secondary structures.}
Many of these alternative structures serve important signalling functions for regulation of gene expression, control of transcription, and protein production, which ensure efficient cellular function. Triplex structures are formed using the unpaired hydrogen bonding sites that remain unoccupied on nucleobases during normal W-C base pairing in B-form DNA. A third strand is able to insert itself into the major groove using these sites, referred to as Hoogsteen binding sites (Figure 1-4). To allow insertion of a third strand, the DNA sequence is usually comprised solely of pyrimidine bases or mostly purine bases with a small portion of pyrimidines mixed in. These structures have been shown to form as frequently as 1 in 50,000 base pairs, and triplex elements are thought to serve as recognition sites for regulatory proteins.

Figure 1 - 5: G-tetrad structure shown by Hoogsteen binding of four guanines and stabilized by a counter ion (K⁺).

Quadruplex DNA structures involve the formation of stable, four-stranded, secondary arrangements from the self-assembly of one strand or by cooperative association of two or more strands. These structures form in G-rich segments and again use Hoogsteen binding sites on guanine bases to assemble into a planar quartet (Figure 1-5). These structures are stabilized by Na⁺ and K⁺ which coordinate to the four
carbonyl oxygens in each G-quartet (Figure 1-5). For long G-rich sequences, many quartets have been found to stack together which underscores the cooperation between π-stacking and cation-dipole interactions. Quadruplexes are commonly found genomically in G-rich telomere regions at chromosome termini. They are thought to play a role in regulation and maintenance of the chromosomal DNA and are essential in preserving genomic integrity. Due to this important role, quadruplex structures have been the target for recent chemotherapeutic advancements. Furthermore, these structures are also present in sequences containing runs of repeated guanines which suggests a role in gene-regulation and recombinatorial processes.

To allow formation of these alternate DNA structures, there is a great deal of inherent flexibility within the oligonucleotide strand. Shown in Figure 1-6 are the bonds that allow flexibility and their corresponding angle notations.

![Figure 1-6: Torsion angles for a nucleotide within the DNA duplex illustrating the possible sites of rotation.](image)

Although each backbone angle is restricted to a discrete range of rotation, the largest rotational freedom occurs at δ and χ torsional angles. The δ angle determines the conformation of the deoxyribose sugar (C2'-endo vs. C3'-endo) and is highly dependent on the glycosidic angle χ, which is defined in terms of four atoms, O4'...
C1'-N9-C4 for purines and contains two domains: syn ($0 < \chi < 90^\circ$) and anti (-120 > \chi > 180^\circ). In B-form DNA, the anti conformation is strongly favored for all bases due to steric interactions between the base and the sugar; however, alternate conformations become favored in A-form and Z-form duplexes. The energy barrier to rotate between syn and anti is different for each DNA base, but guanine possesses the lowest energy barrier due to secondary H-bonding interactions between O5' and NH$_2$ as well as the 5' phosphate.

![Diagram of DNA conformation change](image)

Figure 1 - 7: Rotation around glycosidic bond produces both syn and anti conformations which expose different H-bonding faces. Adduct formation can shift the energetic equilibrium towards the syn conformation which has mutagenic influences.

Preference of the syn conformation amongst guanine rich DNA strands allows the DNA to adopt the Z-DNA structure. Hoogsteen binding requires rotation to a syn conformation, and secondary structures, such as tetrads and quadruplexes that contain...
many Hoogsteen bonds, are G-rich. Furthermore, alterations to DNA bases, which favor
the adoption of a syn orientation, can induce local structural changes, which will
influence toxicological properties of these nucleobase adducts.\textsuperscript{27} It is known that
alterations at the C8 site of guanine favors the syn conformation by 1-2 kcal/mol (Figure
1-7),\textsuperscript{28} which is thought to play a role in mutagenic outcome for compounds that cause
this type of alteration.

The body of knowledge concerning DNA structural variety and function is in a
state of continual growth. However, a commonality to the study of DNA structures, both
new and old, is the importance of maintaining the correct sequences of base pairs
regardless of secondary structure. Sequence alterations and abnormal structures have
shown strong associations with genetic diseases, aging, and physiological
abnormalities.\textsuperscript{29,30}

1.1.3. DNA Aptamers

An interesting application of the regulated, self-associative properties of nucleic
acids has been the development of aptamers. Aptamers are fragments of single
stranded oligonucleotides that take advantage of the molecular interactions possessed
by DNA strands to fold into specific secondary structures. Extensive folding possibilities
allow aptamers to bind a desired molecular target with very high affinity.\textsuperscript{31} Because DNA
has four different nucleobases and any number of sequence variations, the library of
potential aptamers is immense.\textsuperscript{32} With the invention of solid phase DNA synthetic
procedures and the ease of automated selection procedures, it has become possible to
generate a wide library of candidate strands, screen them to a target such as a small
molecule or protein, isolate the desired strand, and characterize the sequence.\textsuperscript{33} Since
every different sequence candidate displays a unique combination of hydrogen bonding,
Van der Waals interactions, loops and bulges, the resulting library displays many different three dimensional attributes that promote interaction with a wide array of targets.\(^{31}\)

![Diagram of TBA aptamer](image)

**Figure 1 - 8: TBA aptamer showing induced folding upon introduction of an appropriate counter-ion. Folding is also achieved by introduction of the substrate.**

Aptamer strands, generated in this way, have shown nanomolar dissociation constants to proteins and are advantageous to antibody development in their ease of preparation, longer shelf life, wider spectrum of action, and greater accessibility potential based on smaller size.\(^{31}\) The aptamer generated for the coagulation protein thrombin is shown in Figure 1-8. This sequence happens to fold into two G tetrad structures with a short T-G-T loop in between. The \(K_A\) for this aptamer in binding thrombin is \(1.6 \times 10^8\) M\(^{-1}\), illustrating the affinity that these molecules possess for their targets.\(^{34}\) Synthetic aptamer oligonucleotides have many functions. These include being used as
aptasensors that release a detectable signal once bound to the target, regulators that bind to a target blocking its interaction with normal DNA, and influencing transcriptional regulation, being placed on chips that are quickly and reliably used as a micro-arrays for small molecules and biomarker detection.

1.1.4. DNA Damage and Genetic Toxicology

DNA is susceptible to chemical reactions that alter its structure in various ways. In general, DNA is particularly vulnerable to attack from electrophiles, reactive oxygen species (ROS), and radicals that will physically damage the helical structure by cutting the sugar-phosphate backbone or creating an abasic site (Figure 1-9). Chemical modification can also occur to the nucleobases themselves by performing oxidation or alkylation reactions (Figure 1-9).

![Figure 1 - 9: Depiction of different types of DNA damage that can occur through reactions with various electrophiles.](image-url)
Species that alter DNA cannot be confined to those agents that originate outside the body from exogenous sources. ROS and alkylating agents occur naturally within cells and are often part of essential signaling pathways. An interesting example of endogenous covalent modification is methylation of DNA. Cytosines are methylated by different methyltransferase enzymes at specific sequences containing CpG. In mammals, between 70-80% of these sequences are methylated. Both a lack of methylation and hypermethylation of these nucleobases have been linked to carcinogenesis, which highlights the importance of these alkylated bases in gene transcription and regulation, as well as the delicate balance required for proper function.

It is easy to associate DNA damage as occurring solely from the exposure to exogenous chemical agents, but endogenous damages are responsible for hundreds of DNA damages per cell per day. Of the different types of DNA damage shown in Figure 1-9, some are more mutagenic than others. This is the result of differences in repair ability, degree of physical distortion to DNA secondary structures, and interactions with replicative enzymes. It should also be emphasized that mutagenic potential does not correlate with carcinogenicity, as many alterations are repaired efficiently by DNA repair enzymes. Furthermore, genetic code redundancy and polymerase fidelity act as a “safety net” for small, single-base alterations. Point-mutations often remain silent and are not observed phenotypically, but will contribute to the overall genetic load of the genome.

1.1.4.1 Covalent DNA damage

One type of prevalent DNA damage, especially pertinent to the work in this Thesis, is chemical reaction between electrophilic species, that are attracted to electron
rich DNA bases and nucleophilic sites on the bases themselves, to form covalent adducts. Many of these molecules react in a site specific manner on individual bases and display sequence specificity within chromosomes. Although some compounds possess electron deficient sites in their chemical structure, many molecules undergo a process of metabolic bioactivation. This enzymatic process attaches a reactive, nucleophilic site on a previously inert molecule to increase polarity and water solubility in an effort to promote excretion (Figure 1-10). An example of this process is the mechanism of toxicity for benzo(a)pyrene, a polyaromatic hydrocarbon (PAH), found in atmospheric pollution from the burning of fossil fuels as well as in burned meats and smoked fish. This compound is metabolized by cytochrome P450 1A1 to a reactive epoxide which forms a covalent adduct with the N2 site of guanine (Figure 1-11). Because of the highly planar, lipophilic nature of the bonded substituent, it prefers the interior of the helix and will flip the orientation of the adducted base from anti to syn, displacing the opposing nucleobase and destroying normal W-C hydrogen bonding interactions.

![Diagram](image)

**Figure 1 - 10**: Bioactivation of xenobiotics through phase I metabolism and attachment of reactive groups to allow phase II metabolism to occur. Phase I conjugates are often responsible for toxicity observed with xenobiotics.
Adduct formation alters the helical structure, disrupts recognition of this base by replicative enzymatic machinery, and can be cytotoxic if not repaired correctly. DNA adducts are classified based on their site of attachment. The electronic nature of the electrophile will determine which site will undergo reactivity on a particular base. In general, guanine is preferentially attacked by electrophilic species due to its electron-rich nature, and several sites on the guanine base have been shown to form different adducts (Figure 1-12). Other bases will undergo similar adduct formation if their nucleophilic sites come into chemical contact with the adducting molecule. Acrylamide, ethylene, and styrene have been shown to undergo metabolic activation to form alkyl epoxide molecules that react at the N7 site of guanine as well as the N6 of Adenine and the N4, O2 sites on cytosine.

Figure 1 - 11: Bioactivation by CYP 450 of PAH to form a diol epoxide and subsequent attack from N2 of guanine to form the N2-PAH adduct.

Although the N7 adduct on guanine predominates in vivo, the other lesions have also shown mutagenic potency. Furthermore, the size and orientation of the incoming
electrophile contributes greatly to the type of adduct observed, as accessible sites located in the major or minor groove will need to come into direct contact to react.\textsuperscript{2} The orientation of the DNA, B-form, A-form, or Z-form has a profound influence on the size and depth of the grooves and, therefore, the attachment sites. It is worth highlighting that PAH's have a larger spectrum of possible attachment sites compared to hydroxyl radicals which tend to react preferentially at one site (C8), depicted in Figure 1-12.

![Diagram of DNA damage mechanisms](image)

Figure 1 - 12: Sites of attack on 2'deoxyguanine preferred by different electrophiles.

1.1.4.2. Oxidative DNA Damage

Oxidative damage to DNA can occur by multiple mechanisms including hydrogen abstraction from the sugar backbone which produces strand breaks,\textsuperscript{53} or from oxidative reactions directly on the electron rich nucleobases.\textsuperscript{54} Hydroxyl radicals (●OH), produced from water radiolysis by ionizing radiation and oxidation products in the electron transport chain (ETC), can react with DNA causing clastogenic damage and point mutations.\textsuperscript{55} The production of deoxyribose radicals from hydrogen abstraction are key intermediates in the formation of strand breaks and are the most severe lesions in
radiation damage to DNA.\textsuperscript{53} These reactions show some preference for specific hydrogen atoms on the deoxyribose sugar based on accessibility to the outside environment and positioning in the helix, which exposes the H atom in the minor or major groove.\textsuperscript{56} Reactions occurring directly to the nucleobases also show base-dependant preference, based on the ease of electron removal which varies between the bases.\textsuperscript{57} The oxidation potentials for the four nucleobases are shown in Table 1-1. The purines have lower oxidation potentials than the pyrimidines, and deoxyguanine has the lowest oxidation potential.

Table 1 - 1: Oxidation potentials of the four nucleobases found in DNA

<table>
<thead>
<tr>
<th>DNA Base</th>
<th>Oxidation Potential (V vs. N.H.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td>1.29</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.49</td>
</tr>
<tr>
<td>Cytosine</td>
<td>1.60</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Although more than 50 oxidatively modified bases have been identified in DNA, the most prevalent lesion is 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) which also serves as the most significant biomarker for oxidative damage in a cell.\textsuperscript{58} The oxidation potential of this species drops further by \( \sim 0.5 \) V, making it a target and providing a driving force for further oxidative damage. 8-oxo-dG has been shown to react with peroxyl radicals (\( \cdot \text{OOH} \)) and singlet oxygen (\( ^1\text{O}_2 \)) to form the more mutagenic guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) lesions shown in Figure 1-13.\textsuperscript{59,60}
Oxidative DNA damage occurs prevalently in the cell and has been implicated in the pathogenesis of mutational fixation, carcinogenicity, and aging. It is an inevitable consequence of cellular metabolism, as well as exposure to ionizing radiation, that produces deleterious effects through a number of different mechanisms. The physiological consequences of this damage are exacerbated by genetic alterations in repair enzymes and lowered anti-oxidant capacity from nutritional deficiencies.

Oxidatively modified nucleobases can alter the structure of DNA, as exemplified by 8-oxo-dG which prefers the more energetically favorable syn orientation when DNA is single stranded. This prevents pairing with natural C and can lead to mutations. Furthermore, some of the modified nucleobases are poorly recognized by repair enzymes. Although 8-oxo-dG paired with C is well recognized and effectively repaired, when 8-oxo-dG is mispaired with A, the base is poorly recognized and therefore not repaired efficiently despite established repair mechanisms. Although cells encounter oxidative damage consistently and have repair enzymes to replace these lesions with correct nucleosobases, it is obvious that oxidative processes can overwhelm repair procedures and provide cytotoxic mechanisms for many DNA damaging molecules.
1.1.5. Genetic Toxicology

The study of the effect of chemicals and physical agents on the hereditary material originated with the work of H. J. Muller, who showed that X-rays could induce phenotypic mutations in fruit flies. Studies continued with work by William Russel who also showed that similar phenotypic mutations could be induced in mammals using X-rays. Interestingly, they were able to elucidate the importance of studying interactions on genetic material before a structure of that material had ever been proposed. Today, we recognize the overwhelming importance of genetic code conservation. The study of potential modifications to this material is of great interest for toxicologists due to its dominant role in disease progression and cancer prevention.

1.1.5.1 Mutations

Spontaneous alterations to DNA sequences or alterations caused by endogenous and exogenous agents are often automatically thought of as mutations. Since these types of processes occur regularly in cells, it is fortunate that this is not the case. Although mutations are key intermediates for evolution and can be beneficial, overabundance or mis-placement of sequence changes will cause cytotoxicity. Cells have extensive abilities to repair alterations; a mutation is, therefore, classified if changes to nucleotide sequences remain unrepaired and undergo rounds of DNA replication that incorporate the mistake into daughter strands. This error is manifested in the subsequent generations of cells and is deemed a mutation. The altered sequence is now the new template for protein production, and incorrectly inserted amino acids will affect interactions between amino acids, altering protein structure, and function.
Chemical mutagens will ultimately induce a mutation by altering the association between the replicative machinery and the template strand. Blocking the reading site, changing the orientation of the base within the helix, or stabilizing the insertion of an incorrect base are three mechanisms that can cause this altered interaction. The ability of carcinogenic adducts to interfere with conformational processes by directly interacting with polymerase enzymes can explain the mutagenic consequences these adducts impose. The carcinogen N-acetyl aminofluorene (AAF) forms a covalent adduct with the C8 position of guanine in DNA. The increased stacking capabilities of the attached moiety and the flexibility of the glycosidic bond allow AAF to flip the orientation of the nucleobase from anti to syn and stack within the helix. AAF acts as a strong block to the polymerase by a mechanism thought to be driven by interactions with hydrophobic amino acid residues located within or near the active site of the enzyme.

These interactions are thought to strengthen the binary DNA-polymerase complex and also preclude binding of a dNTP within the active site. Furthermore, certain amino acids in the binding site are thought to be responsible for forming tight complexes with the templating strand to ensure selectivity for incoming dNTPs. Alterations to these amino acids, namely Tyr 766 or chemical alterations that weaken this important association, have been shown to lead to misincorporations across from AAF adducts.

If the polymerase inserts the same chemical class of base (purine for purine, pyrimidine for pyrimidine), it is called a transition mutation. If a change is made across a chemical class for example, a purine base is switched to a pyrimidine base a transversion point mutation is the result. Since DNA codes for amino acids in groups of three bases called codons and several sequences of three bases can code for the same amino acid, this redundancy allows some point mutations to remain unnoticed as the
The amino acid sequence remains unchanged (Figure 1-14). This is referred to as a silent mutation and will result in a phenotypically normal protein product (Figure 1-14).

**Figure 1 - 14: Single nucleobase changes in the genetic code and resultant amino acids from different mutations. Single amino acids can be changed (missense, silent) or the sequences can be terminated (nonsense).**

Other types of mutations can involve insertion and deletion of nucleotides during replication as a result of a more structurally unique lesion. Insertions and deletion can both lead to frame shift mutations where the entire reading frame of the ribosome is shifted resulting in sequences of several incorrect amino acids instead of a single change. One can imagine that this type of damage is much more serious to the cell since a protein built from this sequence is non-functional instead of simply having an altered function. Finally, a mutation can result in the production of a codon that signals the stop sequence (Figure 1-14). When the enzyme reaches this sequence, it breaks off and the amino acid chain is released. This protein product is truncated and will be completely dysfunctional.
Mutations have occurred in the mammalian genome for millions of years and although site specific mutations are considered harmful and lead to disease states, others cause beneficial genetic changes and have been responsible for important evolutionary events.

1.1.6. Phenols

A class of molecules known to cause prominent genetic damage by a variety of mechanisms are phenols. Phenols are ubiquitous compounds throughout nature, used in a wide range of industrial and pharmaceutical applications, and have several interesting binding and oxidative properties. Phenolic compounds are classified as any molecule with at least one aromatic ring that bears one or more hydroxyl groups.

![Figure 1-15: Examples of different Phenols. A) Phenol B) Gallic acid, an antioxidant found in tea and oak bark. C) Hexahydroxydiphenic acid (HHDP), another antioxidant found in wine and tea. D) Pentachlorophenol, a wood preservative. E) p-cresol, a toxic phenol. F) Vitamin E.](image)

An interesting property of phenols is that they have both pro-oxidant and antioxidant activity. Vitamin E (Figure 1-15, F) is the classic example of a beneficial
A phenolic compound. It quenches free radicals and exhibits anti-oxidant activity in biological membranes by inhibiting lipid peroxidation. It can also chelate catalytic metals, thereby halting their oxidant activity. Several other natural phenolic antioxidants are found in plants and fruits including grapes, olives, and soy. Examples such as gallic acid and HHDP (Figure 1-15, B and C) have been shown to prevent damaging oxidant behaviour and protect cellular organelles, proteins, and DNA by acting as radical scavengers. This protection can help prevent oxidant-mediated diseases like cancer and Alzheimer’s. Unfortunately, in addition to positive anti-oxidant activity, there are many examples of phenols exerting a strong pro-oxidant effect that damages DNA and other cellular components.

Figure 1 - 16: Ambident reactivity of phenoxy radical to form C-linked and O-linked adducts at the C8 position of 2’-deoxyguanosine
Phenol, the simplest member of this class has been shown to possess cytotoxic properties. The wood preservative pentachlorophenol (PCP, Figure 1-15, D) has also shown deleterious pro-oxidant effects. Phenolic compounds found in the oil industry, such as 2,4-dimethylphenol and p-cresol (Figure 1-15, E) are environmental contaminants present in waste water and produce toxicity to several organisms. Other prominent exposures to phenols occur from cigarette smoke, industrial emissions, and some cosmetics. Phenols are thought to exert their toxicity through a number of different mechanisms. Generation of radicals, formation of quinones, redox cycling, and anti-oxidant depletion, as well as oxidation reactions with DNA directly through the formation of adducts, have all been implicated in phenolic toxicity. The mechanism of covalent attachment for phenols is initiated by peroxidise enzymes, CYP 450 monooxygenases, or ROS activation to form electrophilic phenoxyl radicals. These radicals are hydrophobic due to the benzene ring and are relatively stable due to resonance. Phenoxyl radicals also demonstrate ambident reactivity and both C-linked adducts and O-linked adducts have been shown to occur from phenoxyl radical intermediates (Figure 1-16).

1.1.7. Human Metabolism and Endogenous Phenol Production.

An important aspect of the toxicity of phenols is consideration of additional exposure instances from hepatic metabolism of various inactive carcinogens. Metabolism in human hepatic tissues is a dynamic system that relies primarily on a two step process. Many compounds, when they enter the body, are stable, lipophilic, and chemically inert. These compounds must be changed into more hydrophilic forms to prevent diffusion across lipid membranes, accumulation in adipose tissues, and to increase excretion. In general, mammalian systems undertake this task by first attaching a reactive functional group or “chemical handle” onto the molecule in question.
This process, called phase 1 metabolism, facilitates subsequent conjugation with large, water soluble moieties deemed phase 2 metabolism.\textsuperscript{2} The phase 1 processes are carried out in large part by a family of enzymes called cytochrome P450 enzymes (CYP 450). The phase 2 processes are catalyzed by a variety of enzymes and require chemical substrates that are synthesized in the cell or absorbed from the diet. A finite level of these molecules are, therefore, available to detoxify the phase 1 products at any given time. The schematic overview of this process was shown in Figure 1-10.

Stores of phase 2 substrates may be depleted by an overabundance of primary metabolites, an increase in the oxidative stress of the cell, or poor nutritional status. As this system is in constant balance, the accumulation of reactive primary metabolites can lead to toxicity purely based on their increased residency time and probability of undergoing a deleterious chemical reaction with a cellular macromolecule.\textsuperscript{52} An excellent example of this process is illustrated in the toxicity of aflatoxin B1 which forms a reactive epoxide upon phase 1 metabolism and will cause carcinogenicity by covalent attachment to DNA if not adequately conjugated (Figure 1-17).\textsuperscript{52}

It has been well documented that many polyaromatic hydrocarbon molecules, such as naphthalene, biphenyl, and benzene, undergo hydroxylation reactions by interacting with CYP 450 enzymes to form phenols as primary metabolites.\textsuperscript{92} One of the primary metabolites of the biphenyl molecule is the 4-hydroxylated species which has been detected in the urine in addition to the Phase 2 conjugates.\textsuperscript{90} A major metabolite for naphthalene is 2-napthol which has also been detected significantly in the urine.\textsuperscript{91} These primary metabolites are used as biomarkers for exposure to these carcinogens and the ability of these species to survive in the body as bioactivated compounds increases the probability of undergoing deleterious enzymatic activation to the phenoxy radical.
Figure 1 - 17: Aflatoxin B1 bioactivation to form a reactive epoxide that can react with DNA.$^{52}$

Similarly, primary metabolites of polychlorinated biphenyl species (PCBs), which are potent environmental pollutants and known carcinogens, also include hydroxylated species. PCBs have been shown to cause hepatic toxicity and hepatocarcinomas in rat studies.$^{92}$ Furthermore, these species are known to induce the production of the CYP 1A1 enzyme responsible for their own phase 1 metabolism which will increase quantities of hydroxylated species in liver cells.$^{93}$

The potential for these endogenously produced, primary metabolite phenols to undergo phenoxy radical conversion and form covalent DNA adducts remains largely unexplored and will be addressed in detail in Chapter 2 of this Thesis. 2-Naphthol and 4-phenylphenol are metabolites formed in hepatocytes from the metabolism of PAHs and
may contribute a largely unexplored secondary exposure situation not usually considered for these PAH species.

1.2. Tools for Studying Modified Oligonucleotide Interactions

Several different methods are used to validate the successful incorporation of modifications into DNA helices and to study the interactions of these modifications in the duplex.

1.2.1. Mass Spectrometry

Mass spectrometry (MS) is a technique that uses electric or magnetic fields to determine the ratio of mass to charge (m/z) for analyte ions in a sample. To create ions in the gas phase, gaseous or heat stable samples are volatilized and subjected to collision with a charged particle. Thermally labile samples undergo desorption or desolvation ionization using a strong electric field to prevent decomposition. Once formed, ions are analyzed by applying an electric and/or magnetic field that affects ionic trajectory or travel time in a mass analyzer. Ions can therefore be distinguished from each other by differences in size and charge state. Tandem mass spectrometry (MSn) is a technique involving the isolation of a desired ion, followed by collision with an inert gas. This breaks the analyte ions into specific fragments, which permits structural characterization of the parent ion, and analysis of gas phase reactivity. With the invention of softer, less harsh ionization methods such as electrospray ionization (ESI), MS has become useful for the analysis of high molecular weight biomolecules like DNA and proteins. As DNA is a polymeric system with both acidic and basic functional groups, desolvation ionization methods, such as, ESI produce multiply charged
oligonucleotide ions in both polarities relatively easily, with minimal damage to the native structure. The tandem mass spectrometry techniques are used to sequence DNA, while also providing specific structural information about the presence and location of modifications. ESI ionization followed by collision induced dissociation (CID) is employed to analyze modified DNA structures, and verification of a modification location is based on previously established ion chemistry of oligonucleotides in the gas phase, classified by McLucky, and shown in Figure B-6. Tandem MS fragmentation patterns are also used to locate specific ions in complex mixtures by focusing on a particular cleavage pathway or desired fragment from a precursor ion. Selective reaction
monitoring (SRM) and multiple reaction monitoring (MRM) are two experiments that employ tandem MS to identify and quantify desired molecules in a variety of biological media. These molecules are utilized as biomarkers for exposure to toxicants or as a measure of diseased states.

Shown in Figure 1-18 is the mass spectrum of an unmodified oligonucleotide using ESI in the negative mode. Because of the high number of negative ionization sites on the phosphate backbone, DNA easily ionizes in the negative mode and produces a spectrum that contains multiply charged ions of the parent species. The correct mass of the parent molecule is determined by taking the m/z of the multiply charged ion, multiplying by the charge number and adding the protons lost during ionization, or by utilizing deconvolution software. Furthermore, any of these multiply charged ions can be isolated, and fragmented to obtain tandem MS information on the composition of the DNA strand.

1.2.2. Circular Dichroism (CD)

Circular dichroism is a technique that involves shining plane polarized light on a sample and using the optical activity of that sample to measure the differences in absorption of light. CD is very a useful tool to study secondary structures of cellular macromolecules since the degree of absorption of the light beam will vary with differing secondary structures and the relative orientations of the optically active parts of the molecule to each other. DNA is a chiral molecule due to the asymmetry of its sugar-phosphate backbone and the helical arrangement of this natural secondary structures. The more important optical interactions, between neighboring bases, gives the dominant CD peaks for most double stranded DNA. Because of the sensitivity to alterations in these interactions, CD is an advantageous method for tracking perturbations to the
interior environment of the helix and has been used extensively to trace conformational transitions between discreet DNA arrangements.\textsuperscript{104} CD also has the added benefits of being a rapid, sensitive, non-destructive, and relatively inexpensive analytical method.\textsuperscript{105}

The spectra for the different normal forms of DNA are shown in Figure 1-19. It is worth highlighting the differences in CD spectra for B-form DNA vs A-form DNA vs Z-form DNA.\textsuperscript{106} CD spectra for alternate secondary structures like G-quadruplexes are also unique. CD has been used to track quadruplex folding and transitions between interchangeable secondary structures.\textsuperscript{101,107}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure}
\caption{Comparison of CD spectra taken for three different structural adoptions of duplex DNA strands.\textsuperscript{104}}
\end{figure}

Comparisons in the shift of the peak maxima, the intensity of these peaks, and induced CD bands have been shown to correlate with the amount of stacking interactions between bases,\textsuperscript{108} structural changes like DNA bending and kinking,\textsuperscript{109} and the orientation of DNA adducts within the helix.\textsuperscript{110} Using CD analysis enabled the
acquisition of some structural information on the secondary structures adopted by the modified helices under study, without destroying the sample.

1.2.3. Melting Temperatures \( (T_m) \)

Another important analytical tool in the analysis of nucleic acids involves the measurement of helix melting temperatures \( (T_m) \). This technique is based upon the principle that as two strands of DNA break apart (denaturation) and reanneal (hybridization), specific changes occur in the molecular absorbance, due to variations in viscosity, optical rotation, and stacking interactions.

![Figure 1](image.png)

**Figure 1 - 20:** \( T_m \) melting curve taken by monitoring the UV absorbance values at 260 nm. The inflection point of the graph corresponds to the \( T_m \) value for the DNA duplex.

While observing the absorbance at this specific wavelength, it is possible to follow denaturation and hybridization processes with a UV-vis spectrophotometer. As the DNA strands randomly come into contact with one another and begin forming hydrogen bonds with complimentary base pairs of the opposing strand, there is a measureable
decrease in absorbance at 260 nm which is called the hypochromic effect.\textsuperscript{111} Each strand studied has its own characteristic curve which is obtained by repeated heating and cooling of the DNA solutions and is shown in Figure 1-20. The melting temperature or $T_m$ is the temperature at which half the strands are annealed and half are still denatured. This value is calculated by taking the derivative of absorbance vs temperature relationship or by finding the point of inflection on the graph. This value is used to compare different duplexes as higher melting temperatures represent more stable helices. For example, a strand rich in guanine-cytosine (G-C) base pairs provides a much higher $T_m$ when compared to a strand of adenine-thymine (A-T) base pairs of the same length.\textsuperscript{112} Increases in total hydrogen bonds for the G-C strand allows more stable duplexes to form which are more difficult to denature. Modified DNA strands can, therefore, be compared to the native sequence by comparing the change in the $T_m$ value which provides insight into the ability of the modification to stabilize or destabilize the local helical environment of DNA.\textsuperscript{113}

1.3. Overview of Synthetic Strategies Employed

1.3.1. Suzuki Miyaura coupling to obtain C8-Aryl-dG Adducts

A common synthetic strategy, employed by many members of the Manderville laboratory to obtain the large family of C8-aryl-dG adducts studied, utilized the Suzuki-Miyaura coupling approach. The reaction won Akiro Suzuki the Nobel Prize for chemistry in 2010 because of the broad range of applications to form carbon-carbon bonds. The reaction is a palladium-catalyzed cross-coupling between an organoboron compound and an organic electrophile like a halide or triflate in the presence of base to form a C-C bond.\textsuperscript{114} The mechanism for this reaction is shown in Scheme 1-1 and involves three
major steps. A ligand (L), usually triphenyl phosphine or a derivative thereof, activates the palladium. The first step of the cycle involves coupling of the palladium catalyst with a halide (X) by way of an oxidative addition to form an organopalladium complex, R-Pd(II)L2-X. Transmetallation with the organoboron compound affords the complex, R-Pd(II)L2-R' and the boron-halide leaves. The final step is a reductive elimination to give the desired product, and the palladium(0) is available for another catalytic cycle.

Scheme 1 - 1: Palladium catalyzed Suzuki Miayaura cross-coupling reaction cycle.
Although the exact mechanism of the transmetallation is still not completely understood, the base added to the reaction is thought to have three roles in the cycle according to Le Duc and co-workers.\textsuperscript{115} Base was shown to accelerate the formation of the palladium complex \( R \text{-Pd(O)L}_2 \text{-X} \), promote the formation of the trialkyl borate, polarize the organic ligand to aid transmetallation, and increase the rate of the reductive elimination step by promoting a catalytic pathway involving pentacoordinated, anionic palladium complex.\textsuperscript{115}

The C8-aryl adducts used in Chapters 3 and 4 were synthesized according to a procedure presented originally by Shaughnessy and co-workers.\textsuperscript{116} This procedure used an aryl boronic acid (\( \text{Ar-B(OH)}_2 \)) as the organoboron component and 8-bromo-2'-deoxyguanosine (8-Br-dG) as the organic halide. 8-Br-dG is synthesized by brominating the C8 position of dG using NBS (Scheme 1-2).\textsuperscript{117} The Suzuki coupling procedure, using the newly synthesized 8-Br-dG and the appropriate boronic acid (\( \text{Ar-B(OH)}_2 \)), was carried out with a water soluble Pd catalyst (\( \text{Pd(OAc)}_2 \)) in the presence of a water soluble phosphine ligand, 3,3',3''-Phosphanetriyltris(benzenesulfonic acid) trisodium salt (TPPTS) in an \( \text{H}_2\text{O:ACN} \) solvent system.

\textbf{Scheme 1 - 2: Synthesis of 8-Br-dG}
This method produced ideal solubilities for the nucleoside component, yielded the desired adducts in adequate amounts, and prevented the use of protection-deprotection steps for the nucleoside. Protection of the 3'- and 5'-OH groups would be necessary by attempting the Suzuki coupling under conventional anhydrous conditions (Scheme 1-3).

**Scheme 1 - 3: Suzuki Miyaura coupling reaction to afford C8-aryl-dG adducts**

```
\[
\begin{align*}
\text{Br} \quad \text{N} \quad \text{N} \quad \text{NH} \\
\text{HO} \quad \text{O} \quad \text{OH} \\
\text{8-Br-dG}
\end{align*}
\]

\[
\begin{align*}
\text{Ar-B(OH)}_2 & \quad \text{Pd(OAc)}_2/\text{TPPTS} & \quad \text{Na}_2\text{CO}_3 \quad 2:1 \text{H}_2\text{O}:\text{CH}_3\text{CN} \\
& & 80^\circ\text{C}, 4h \\
\text{C8-aryl-dG}
\end{align*}
\]

\[
\begin{align*}
\text{SO}_3\text{Na} \\
\text{TPPTS} = \quad \text{SO}_3\text{Na} \\
\text{NaO}_3\text{S}
\end{align*}
\]

**1.3.2. Synthesis of O-linked Nucleosides**

O-Linked C8-dG adducts were not synthesized by Suzuki coupling since an ether bond was desired, not a carbon-carbon bond. The methodology utilised for this reaction was proposed in 2011 by Dahlmann and Sturla and involved synthesizing an O\(^3\), O\(^5\), and O\(^6\) protected 8-Br-dG analogue.\(^{118}\) An S\(_\text{NAr}\) displacement reaction was established using
toluene, dimethoxyethane (DME), or xylenes as solvents. Successful reactions were also performed both in air and under inert atmosphere.\textsuperscript{118} The reaction was optimized and maximum yields were obtained while using a base (K$_2$CO$_3$) to create the phenolate anion in xylenes at 135 °C (Scheme 1-4).\textsuperscript{118}

**Scheme 1 - 4: Synthesis of O-linked C8-dG adducts proposed by Dahlmann and Sturla.\textsuperscript{116}**

![Scheme 1 - 4: Synthesis of O-linked C8-dG adducts proposed by Dahlmann and Sturla.\textsuperscript{116}](image)

Work by Michael Kuska in the Manderville laboratory further modified this reaction procedure, avoiding low-yielding removal of the benzyl group at O6, by protecting it with trimethylsilylethanol (TMSEtOH) which is removed by TBAF.\textsuperscript{119} The overall synthetic strategy was reduced by one step. The synthesis of certain O-linked adducts for this project, the chlorinated derivatives, proved difficult due to low nucleophilicity and lipophilicity of the chlorinated phenol reactants, which hindered their reactivity and prevented extraction with aqueous washes. Modifications from the Kuska procedure included longer reaction times and rigorous drying of all reaction components to minimize nucleophilic competition from water. Furthermore, a purification step was employed prior to deprotection which lowered product yields but improved product isolation dramatically. The complete synthetic pathway is shown in Scheme 1-5.
1.3.3. Incorporation of Modified Nucleosides into DNA

Studies on modified nucleosides convey some structural information but properties can change once these lesions are inserted into a helical environment. Therefore, to gather more biologically relevant information and to study these modifications further, certain O-linked C8-dG adducts were inserted into DNA. Currently, there are four common methods to incorporate modifications into an
oligonucleotide. They include synthesizing a modified phosphoramidite and using automated solid-phase synthetic techniques; post-synthetic modification of DNA bearing a convertible nucleoside; post-synthetic modification of DNA by electrophilic attack; and incorporation of a modified nucleoside with an enzyme.\textsuperscript{121,122} Phosphoramidite chemistry and solid phase synthesis is the current industry standard and utilizes methods developed by Beaucage and Caruthers.\textsuperscript{123,124} This technique can synthesize oligonucleotides ~ 200 nucleobases in length.\textsuperscript{125} The Manderville laboratory previously attempted post synthetic Suzuki coupling procedure with some success; however, this technique possessed limitations when it was desirable to incorporate multiple modifications into one strand and yields were poor when longer strands were attempted.\textsuperscript{126} Therefore, recent efforts have focused mainly on the phosphoramidite/solid-phase synthesis approach.\textsuperscript{127}

Phosphoramidites are the monomeric units of nucleotides that contain a phosphoramidite group attached to the 3'-OH of the 2' deoxyribose sugar. The general synthetic strategy is shown in detail in Scheme 1-6 and typically involves protection of the exocyclic amino group at the N2 position with N,N-dimethylformamide (DMF).\textsuperscript{128,129} Other groups such as isobutyric anhydride and 9-fluorenlymethoxy carbonyl chloride (Fmoc-Cl) have also been used with good success.\textsuperscript{130} Subsequent protection of the 5'-OH group with 4,4'-dimethoxytrityl group (DMT) is the standard approach and was used exclusively for this project with excellent results.\textsuperscript{131} However, recent efforts by Mike Sproviero of the Manderville laboratory developed the use of 2,7-dimethyl pixyl (DMPx) group as a more acid labile protecting group better suited for the acid sensitive, C-linked aryl adducts.\textsuperscript{127} The final step uses a phosphitylating agent; in this case, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite coupled at 3'-OH, to afford the fully protected phosphoramidite that is loaded onto the solid phase DNA synthesizer.\textsuperscript{123,124}
The automated oligonucleotide synthesizer utilizes both unmodified and modified nucleoside phosphoramidites as reagents and undergoes a precise series of chemical reactions involving repeated rounds of deprotection, coupling, capping, and oxidation which are described briefly below (Scheme 1-7).132

The initial protected nucleoside is anchored to a solid support, typically composed of borosilicate glass, by a succinyl linker to the 3’OH group, with 5’OH protected by a dimethoxytrityl (DMT) group.
The first reaction in the cycle is treatment with dichloroacetic acid (DCA) to cleave the DMT group and expose the 5’OH site on the anchored nucleoside (1). The machine injects the phosphoramidite corresponding to the next base in the sequence, and treatment with tetrazole catalyzes a condensation reaction between the 3’-β-cyanoethylphosphoramidite of the incoming nucleoside with the free 5’OH (2). A capping reaction (3a) using acetic anhydride blocks any remaining free 5’OH groups that have not reacted to ensure the uniformity of strand construction. In order to produce the
desired phosphate linkage, the phosphite ester is oxidized with iodine (3b). The cycle then repeats by deprotecting the next 5’OH group with DCA and is repeated to the desired length of oligonucleotide with typical yields of 95-99% for each coupling step. Following the synthesis of the strand, the bead is treated with concentrated aqueous ammonium hydroxide at 55°C for several hours to hydrolyze the linker and remove any of the remaining protection groups (4). HPLC purification on a semi-preparative scale with a RP C-18 column using a mobile phase of 95% triethylammonium acetate (TEAA): 5% acetonitrile (ACN), with an increasing gradient of 30% triethylammonium acetate: 70% acetonitrile for elution.

1.4. Research Focus

Current and past work in the Manderville laboratory has focused on the study of C8-aryl-dG adducts. This area of research originated from findings that electron deficient phenoxy radical species derived from pentachlorophenol (PCP) produced an O-linked covalent adduct with dG. Work on the chlorophenolic mycotoxin Ochratoxin A also showed formation of a C-linked C8-dG adduct through a similar, radically mediated, covalent attachment mechanism. Further studies examined mechanisms of phenoxy radical production and covalent attachment to deoxyguanosine (dG) at the C8 site. These studies yielded important information about the reactivity of carbon radicals, as well as, metal binding potential and electrochemical characteristics of C-linked phenol adducts. The chemistry used to create C-linked dG adducts was then utilized to synthesize a family of adducts containing interesting and potentially useful fluorescent properties.

Although preliminary data of the nucleobases themselves gave many interesting results, efforts in the Manderville laboratory shifted to incorporate and study these
modifications in the DNA environment. Early attempts to use a Suzuki coupling method site specifically on the strand showed some success but were technically difficult reactions, gave small amounts of product, and had a narrow range of applications. Overcoming early synthetic challenges, due to hydrolytic instability of the adducts and successfully synthesizing modified phosphoramidites on a large scale, was extremely significant for the Manderville laboratory because it expanded the family of adducts available for study, and allowed for site specific incorporation into a host of different DNA strands by solid-phase synthesis.

The Manderville laboratory began inserting adducts into biologically relevant systems to analyze the conformational and structural preferences of lesions from various toxicants including phenols. Synthetic and analytical techniques developed in the laboratory also promoted the incorporation of modifications into functionalized DNA molecules for potential industrial applications.

The goals of this Thesis include the expansion of previously successful work in the Manderville laboratory and providing new applications for current work.

As structural information on the class of \( O \)-linked C8-dG adducts is largely absent, and these adducts may play an important role for toxicity of some suspected phenolic carcinogens, it is essential to provide fundamental data on the conformational preference of these adducts in a DNA environment. We believe that \( O \)-linked adducts at the C8 site of dG will dramatically alter the conformation of the adducted base in a duplex and may provide mechanistic information on mutagenesis. Chapter 2 addressed this goal by examining a family of interesting, biologically relevant, \( O \)-linked C8 dG adducts within the NarI recognition sequence. Upon duplex formation, the conformational preference of these adducts was investigated by using several different
complementary strands and measuring molecular interactions. Structural characteristics of duplexes induced by these adducts were elucidated using circular dichroism (CD) and thermal melting ($T_m$). Structural influence of ring size, flexibility, and degree of chlorination were three properties studied.

As in vivo evidence for formation of $O$-linked adducts from chlorinated phenols is lacking, we believe that mass spectrometry (MS) is the method of choice for biomarker detection of these adducts and would provide fragmentation patterns to analyze and quantify DNA lesions after exposure. MS was used in Chapter 2 to examine hydrolytic stability of $O$-linked polychlorinated nucleoside adducts in the gas phase and to characterize fragmentation patterns of the adducts, both as nucleosides and nucleotides. Unique fragments were used as effective dose biomarkers for polychlorinated phenol exposure, increasing the biological relevance of these covalent lesions.

$C$-linked C8-guanine adducts from phenolic toxins have been studied previously in the Manderville laboratory, and it was found that these adducts caused a decrease in oxidation potential which facilitated further oxidative processes at the adduct site.$^{145}$ A common oxidative pathway for phenolic species is the ability to form catechols and quinones through reaction with peroxidase enzymes and radical species produced in situ. Quinones are well known, popular targets for attack by nucleophilic sites in DNA and proteins.$^{88}$ We believe that phenol $C$-linked adducts ($p$-PhOH-dG) will undergo hydroxylation to form a catechol and subsequently a quinone adduct at the C8 site. Furthermore, the resulting quinone adduct would be susceptible to nucleophilic attack, providing another possible mechanism for phenolic toxicity though cross-link formation. Chapter 3 focused on secondary oxidative properties for $p$-PhOH-dG and expanded upon the oxidative reactivity of $C$-linked phenol adducts. Formation of a quinone in DNA would create a susceptible site for nucleophilic attack within the helix, affording DNA-
DNA or DNA-protein cross-links and increasing the toxicological significance of this oxidative pathway. The C8-catechol adduct was synthesized to explore potential secondary oxidation pathways for C-linked phenol adducts. In addition, quinone formation from secondary oxidative reactions was confirmed by trapping studies conducted with N-acetylcysteine (NAC) demonstrating reactivity with sulphur nucleophiles.

With the development of functional oligonucleotides such as aptamers for a large range of diagnostic, sensory, and small molecule binding applications, the incorporation of modified nucleobases that increase binding interactions, stabilize secondary structures and confer desirable functionality, is a new field of research with industrial applicability. Furans form a ring-opened dialdehyde species from oxidative activation, investigated by Peterson and co-workers, which reacted with several amino acids to form covalent linkages.\textsuperscript{154} Based on these studies and the creation of a reactive nucleoside by phenol adduct oxidation (Chapter 3), we believe that incorporation of a furyl modification into an aptamer would provide a selective site that could form a covalent linkage with a free amine group on a protein target. The research described in Chapter 4 implemented oxidatively active nucleoside modifications to an aptamer, which formed covalent linkages between the aptamer and its protein target. Biological and industrial applications for this proof-of-concept would include unambiguous identification of unknown molecular targets for aptamers which would aid in assigning biological consequences from target interactions. Furthermore, covalent linkages to targets could increase therapeutic effects with aptamers pertaining to target inhibition.

1.5. References


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determining the guanine plus cytosine content of DNA. In *Methods in


Chapter 2.

Structural Influences and Gas-Phase Fragmentation of O-Linked C8 Guanine Adducts from Phenolic Carcinogens
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- Article direct link: http://pubs.acs.org/doi/abs/10.1021/tx400252g


- Article direct link: http://pubs.acs.org/doi/abs/10.1021/jo401122j


2.1. Introduction.

As discussed in Chapter 1, phenols are ubiquitous compounds that possess many biological properties including toxicity.\textsuperscript{1} Human exposure to phenolic toxins occurs predominately through industrial activities, tobacco smoke, and inhalation of polluted air.\textsuperscript{2,3,4} Phenol exposure can occur through direct contact with the parent phenol or through exposure to aromatic compounds including benzene, polyaromatic hydrocarbons (PAHs),\textsuperscript{5,6} and polychlorinated biphenyls (PCBs),\textsuperscript{7,8} that have been classified by the International Agency for Research on Cancer (IARC) as human carcinogens.\textsuperscript{9,10} These prominent environmental contaminants are well known to undergo metabolism, producing phenols as phase 1 products (Figure 2-1).\textsuperscript{11}

![Chemical diagram](attachment:image.png)

**Figure 2 - 1. Bioactivation of several common environmental pollutants to produce phenols as phase 1 products.**
Aromatic hydrocarbons and polychlorinated biphenyls (PCB) are both known to undergo P450-mediated hydroxylation to generate phenolic metabolites. Phenols, generated endogenously through cytochrome P450-mediated pathways, serve as biomarkers of exposure to both PAHs and PCBs (Figure 2-1).

Chlorophenols are a class of compounds used historically as biocides and wood preservatives and are persistent organic pollutants (POPs). Although use of these compounds has been tightly regulated in many countries since the 1970’s, their lipophilic nature encourages contamination of groundwater, soil samples, and various food chains. Pesticide runoff, industrial waste, and leaching from contaminated sites are the main sources of contamination. Human exposure to chlorophenols occurs primarily by drinking contaminated water sources or living within close proximity to contaminated sites. Direct exposure also occurs through contaminated production containers in food preparation (wine barrels) and consumption of contaminated foodstuffs. Additional exposure can occur through hydroxylation of hexachlorobenzene which, similar to PAHs and PCBs, is the primary metabolic step for these pollutants and may contribute significant indirect exposure to polychlorinated phenols in vivo (Figure 2-1).

Toxicity of phenols stems from their oxidative metabolism that can generate hydroquinone/quinone redox pairs, ROS, and phenoxyl radicals. The generation of hydroquinone/quinone species and redox cycling by phenols will be fully addressed in Chapter 3, since human exposure to DNA-damaging radicals and quinone electrophiles is associated with cancer and aging. Despite genotoxicity of many phenols in vitro, phenol itself lacks carcinogenicity in animal models. This has been attributed to sulfation and glucuronide conjugation in periportal tissues, which detoxifies the parent phenol before reaching the liver, leaving little available for oxidation within hepatocytes. Therefore, phenols created endogenously through hepatic P450-

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mediated metabolism are in close proximity to oxidizing enzymes which can produce damaging metabolites. This proximity increases the chances of creating deleterious, reactive species in cells.\textsuperscript{32,33} One could argue that this secondary exposure situation may be more toxicologically significant than direct contact to the parent phenols themselves.

A prominent genotoxic mechanism for phenols is attributed to their ability to form phenoxy radicals that can attach covalently to the C8-site of 2'-deoxyguanosine (dG) to afford C8-dG adducts.\textsuperscript{34-38} Due to the ambident electrophilicity of phenoxy radicals, both carbon and oxygen (C vs O) C8-dG adducts have been observed.\textsuperscript{1,39} The phenolic C-linked C8-dG adducts belong to a larger class of C8-aryl-dG lesions produced by a number of chemical mutagens that include PAHs,\textsuperscript{40,41} estrogens, nitroaromatics, and arylhydrazines. These adducts are discussed extensively in Chapter 3 of this Thesis.\textsuperscript{42} Phenolic O-linked C8-dG adducts, which is the focus of this chapter, are structurally similar to the corresponding nitrogen (N-linked) adducts produced by arylamine\textsuperscript{43-46} and nitroaromatic carcinogens.\textsuperscript{46} O-linked adducts have been shown to occur from \textit{in vitro} studies of some chlorinated phenols\textsuperscript{36} and are thought to play a role in the mutagenic effects observed for these compounds.\textsuperscript{47,48}

Polychlorinated phenols are known toxicants and possible human carcinogens.\textsuperscript{47} There are several mechanisms of toxicity for PCP including mitochondrial energy depletion,\textsuperscript{49} promotion of oxidative stress through production of reactive oxygen species (ROS),\textsuperscript{50} and metabolism, which produces quinone electrophiles\textsuperscript{48,51,52} and phenoxy radicals. These reactive species form covalent adducts with DNA (Figure 2-2).\textsuperscript{36} Phenoxy radicals are stabilized by the mesomeric effect of Cl-substitution, which also inhibit removal of phenoxy radicals via dimerization/polymerization reactions in environmental systems.\textsuperscript{53} Consequently, chlorinated phenoxy radicals possess greater lifetimes in aqueous solution than unsubstituted phenoxy radicals.\textsuperscript{53} Long-lasting,
persistent, pentachlorophenoxy (PCP) radicals have been shown to form on the surface of transition metal particles in soil and survive for years in contaminated sites.\textsuperscript{54} Cl-Substituted phenoxy radicals are also produced endogenously by peroxidase enzymes\textsuperscript{55,56} and, as mentioned, have demonstrated the ability to bind covalently to the C8-site of dG to afford O-linked C8-dG adducts (Figure 2-2).\textsuperscript{35,36}

![Chemical diagram](image)

**Figure 2 - 2**: Competing covalent toxic pathways of polychlorinated phenols in DNA. Oxidation can form the quinone which reacts covalently to form a benzetheno adduct or phenoxy radical to form a C8 O-linked adduct.

We were interested in studying the effects of O-linked adduct formation at the C8 position of guanine in an attempt to elucidate a possible mutagenic mechanisms for compounds such as PCP, but also to conduct a structure-activity examination to study the influences of structural differences in O-linked adducts to their potential mutagenic potency. The literature is suggestive that some inferences can be drawn between an adducts’ ability to change the local helical structure of DNA and its mutational ability.\textsuperscript{57} A large body of work exists on structural studies for N-linked adducts derived from amine
carcinogens, but the literature is devoid of information on O-linked adducts. We were, therefore, interested in probing the behavior of O-linked adducts derived from environmentally persistent phenols and phenols formed as secondary metabolites of suspected PAH carcinogens.

Chapter 1 stated that modifications of dG at the C8 position is known to affect the barrier between the syn and anti conformation for the free nucleoside and within the DNA duplex. Modification at the C8 site of 2'-deoxyguanosine favors formation of the syn orientation. Computational analyses, done by Wetmore and colleagues, demonstrated a ~25 kJ/mol preference for the syn orientation for a group of C8-aryl-dG adducts. Steric interactions between the sugar and the bulky adduct moiety, as well as an intramolecular hydrogen bond between 5'OH--N3, were hypothesized to contribute to this observed preference (Figure 2-3).

![Steric Interactions and H-Bonding](image)

Figure 2 - 3: Factors that influence syn or anti preference of adducted nucleosides. Steric interactions and H-bonding play a major role in orientation preference.

When these types of studies are extended into the DNA helical environment, preferences for either the syn or anti conformations become more complicated and result in three major possible conformations which exist in equilibrium. The major energetic factors that affect preference for one conformation over another include: the
stability of the normal Watson-Crick H-bonding interaction vs. possible Hoogsteen H-bonding, the ability of the adduct moiety to π-stack within the helix between neighboring bases, and the hydrophobicity of the adduct moiety in attempting to minimize solvent interactions. All of these interactions will be influenced by adduct size, planarity, and presence of functional groups.

Figure 2-4: N-linked adducts formed at the C8 site of guanine by several aryl amine carcinogens.

Arylamine and PAH carcinogens represent an important class of environmental mutagens that produce N-linked DNA adducts in a chemically predictable fashion through metabolic activation to the nitrenium ion. There has been a great deal of research into conformational heterogeneity for these N-linked C8-dG adducts. Four examples shown in Figure 2-4 are 2-(acetylamino)fluorene (AAF-dG), 2-aminofluorene (AF-dG), 4-aminobiphenyl (ABP-dG), and aniline (AN-dG). Through the use of fluorescence, 1H NMR, 19F NMR, crystallographic analysis, and circular dichroism
three main conformations were predicted for C8-arylamine dG adducts within the duplex. The first, called the “B-type” (B), is the least perturbing conformation and projects the adducted moiety into the major groove, exposing it to the aqueous environment. The modified guanine retains the anti conformation, and normal \( W-C \) hydrogen bonding interactions occur with the opposing strand. A base-displaced "stacked" (S) conformer is the result of the modified guanine residue rotating into the syn orientation, rupturing the \( W-C \) hydrogen bonding interactions, and flipping the adduct moiety into the interior of the helix. The \( W-C \) face of guanine projects into the major groove, and the opposing base is flipped out of the helix. This orientation forces stacking of the adduct moiety with neighbouring bases. The final conformation, deemed "wedge" (W), typically arises when the modified guanine is mispaired opposite another purine base. The modified guanine adopts a syn conformation, but unlike the S conformer, the guanine portion remains stacked within the helix, and the adducted moiety is inserted into the minor groove.

The propensity of an adduct to adopt one conformation over another will be a direct result of the energetic factors mentioned above, and has been shown to also depend strongly on sequence. Studies on a variety of lesions have shown that adducts are capable of forming more than one conformer in DNA. The three conformers are depicted in Figure 2-5, adopted from a figure originally designed by Michael Kuska, in the Manderville laboratory.
Figure 2 - 5: Three conformations adopted by *N*-linked adducts in DNA. Also shown is the *syn/anti* orientation of the adducted guanine base. The *N*-linked adduct depicted is 4-aminobiphenyl (ABP).^{77}

Shapiro et al. studied the effects of ring size on the conformational equilibrium for *N*-linked aromatic adducts using C8 modifications by *N*-linked aniline (AN-dG), aminofluorene (AF-dG), and aminopyrene (AP-dG). These adducts were inserted into a variety of sequences and studied by $^1$H NMR, supplemented with computational analyses.^{65} AN-dG was found to adopt exclusively the B conformation, while AP-dG had a significant preference for the S conformer, with small amounts of W.^{65} However, the
AF-dG modified duplex showed all three conformations B, W, and S, with a majority (70%) existing as the S conformer. This study demonstrated that larger, increasingly planar ring systems, preferred the interior of the helix, which resulted in the S conformation and more syn preference.

A simplified conformational analysis using fluorinated substrates and $^{19}$F NMR by Zhou et al. was also used to study this conformational heterogeneity. C8 N-linked adducts from fluoroaminophenyl modified dG (FAF-dG) and C8 fluoroaminobiphenyl modified dG (FABP-dG) were used to demonstrate that FABP-dG adopted the B conformation exclusively, while FAF-dG showed a 55:45, S:B ratio. These results were in agreement with previous $^1$H NMR structural studies.

Cho et al. published an in-depth analysis of several polyaromatic amine adducts based on CD evidence and $^{19}$F NMR resonances within an oligonucleotide. This work substantiated previous studies, but found that the conformational preferences of N-linked adducts varied depending on flanking sequences. For example, the conformers of FAF-dG were found to adopt a ratio of 5:65:30 (B:S:W) with two cytosines flanking the modified base, and a ratio of 5:30:65 with a thymine and adenosine flanking the lesion. These results demonstrated the dynamic nature of an adducts’ conformational equilibrium, and the dramatic influences of flanking sequences on conformational preference.

Cho and colleagues also showed that not only the nearest neighbors, but also the next nearest neighbors, have a strong influence on the S/B ratio. For example, the S-conformer is predominant (about 70%) in a sequence (CCATCG*CTACC) in which the nearest neighbors are identical as the G$_3$ NarI sequence context (5’-CG*C-3’), but the next nearest neighbors are different. These impacts have additionally been observed for PAH-adducts.
While much of the evidence concerning conformational equilibria focuses primarily on N-linked adducts, C-linked adducts at the C8 position of guanine have been predicted to adopt similar orientations based on their chemical properties.\textsuperscript{73,74,81} Molecular dynamics (MD) simulations, performed by the Wetmore laboratory, calculated a similar energy for the \textit{syn} and \textit{anti} orientations of both \textit{para} and \textit{ortho} C-linked phenolic adducts.\textsuperscript{59} They predicted several possible orientations within the DNA environment due to this energetic indifference.\textsuperscript{59} In addition to MD data for \textit{ortho} and \textit{para} phenol adducts, CD experiments on the 8-benzothiophenyl-dG adduct produced an induced CD band corresponding to the absorbance maximum of the benzothiophene moiety.\textsuperscript{82} This result was interpreted as contribution from benzothiophene within the helix, thereby rendering this adduct in the S conformation.\textsuperscript{82} Further investigation into the structural preferences of phenolic, furanyl, and benzothiophenyl C-linked modified duplexes deduced that conformation was heavily influenced by the opposing base. Adducts folded into a duplex opposite the correctly paired C were found to prefer \textit{anti} B-type structures; whereas, forming duplexes with a mismatched G across from the lesion produced W conformations (Figure 2-6).\textsuperscript{83}

The sensitivity of conformational equilibrium adopted by C8 adducts to adduct structure, flanking sequences, and opposing bases illustrates the variety of impacts that an adduct can exert on the local DNA structure. The range of different mutational events caused by similar adducts can be explained by the adoption of promutagenic vs. nonmutagenic conformers. Promutagenic conformations of C8 N-linked arylamine adducts are believed to be the \textit{syn} conformations (S, W), as relative mutagenicity parallels conformational preference for these structures.\textsuperscript{67} Specifically, AAF modified duplexes which adopt the \textit{syn} conformation cause frameshift mutations.\textsuperscript{72,84} ABP
modified duplexes, which show some preference for both syn/anti conformations, are less mutagenic but have been observed to cause G → T transversions.\textsuperscript{72}

![Figure 2-6](image)

**Figure 2 - 6**: MD simulations showing C-linked benzothiophenyl adduct in the B conformation across from C and W conformation opposite G. Reproduced with permission from reference \textsuperscript{81}.

Polymerase studies have found sequences which are more susceptible to producing frameshift mutations.\textsuperscript{85} The sequence 5'-G\textsubscript{1}G\textsubscript{2}CG\textsubscript{3}CC-3', called *Nar*I, forms the recognition sequence of the *Nar*I type II restriction endonuclease and is a hot spot for frameshift mutations\textsuperscript{85} due to a GC repeat that may be an important contributor towards the development of carcinogenesis.\textsuperscript{81} Modification at the C8 position of the G\textsubscript{3} guanine by bulky arylamines has shown -2 frameshift mutation induction.\textsuperscript{43,45} This observation has been explained using the slippage model, first developed by Streisinger, and shown in Figure 2-7.\textsuperscript{86}
Studies by Rizzo used the arylamine carcinogen, 2-amino-3-methylimidazo[4, 5-\(f\)]-quinoline (IQ). These experiments determined that when Narl modified at G\(_3\) by IQ was subjected to polymerase extension studies, replication proceeded normally until the modified base was encountered. The natural C pair was incorporated; however, it paired with the guanine located two bases further down the template sequence in the 5' direction, causing a "slip". This misalignment of base pairing can happen in regions of base repeats, and the effects are exacerbated by adducts that can stabilize this mismatch by stacking. IQ adopts the S conformation, which allows for stabilization of the bulge, by providing π-stacking interactions within the gap.\(^{48}\) Two base pairs are now absent from the replicated strand and will produce a −2 frameshift upon translation. As this interaction requires a stabilization from stacking interactions, the propensity of \(N\)-linked arylamine adducts to form −2 frameshift mutations has been correlated to their
conformational preference for the S conformer which inserts the lesion into the DNA, and stabilizes this gross misalignment.\textsuperscript{45,87} The ability to study −2 frameshift mutations in conjunction with other sequence effects, as well as, the role of \textit{NarI} as a mutation hot spot were the primary reasons for use of this sequence for DNA studies presented herein.

![Figure 2-8: MD simulations of O-linked nucleoside adducts derived from phenol (\textit{PhO}dG) and pentachlorophenol (\textit{PCPO}dG) showing strong syn orientational preference and stabilizing H-bonds. Also depicted is a relatively planar structure for \textit{PhO}G and a skewed structure for the \textit{PCPO}dG adduct. Reproduced with permission from reference \textsuperscript{88}.](image)

Although little is known about \textit{O}-linked adducts that form at the C8 site of guanine, preliminary MD studies on \textit{O}-linked nucleosides demonstrated a strong syn preference, encouraged by adduct bulk, and stabilized by H-bonding between H—O5—N3 (Figure 2-8).\textsuperscript{88} Kuska and colleagues in the Manderville laboratory recently incorporated C8-phenoxy-guanine (\textit{PhO}G) into the G\textsubscript{3} position of the 12-mer \textit{NarI} recognition sequence (5'-'CTCG\textsubscript{1}G\textsubscript{2}CG\textsubscript{3}CCATC-3') to study conformational influences of the simplest aryl \textit{O}-linked adduct.\textsuperscript{77} On the basis of circular dichroism (CD), NMR experiments, and molecular dynamics (MD) simulations, the \textit{PhO}G lesion was predicted

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to adopt the major groove B conformation opposite C (Figure 2-9). These findings correlated with the conformational preference for the corresponding single-ring 
linked C8-dG adduct produced by aniline. On the basis of this comparison, the PhO
lesion was predicted to be weakly mutagenic. Structural similarities between the O
linked adducts and corresponding N-linked adducts, produced by arylamine and nitroaromatic carcinogens, are very useful to aid predictions on structural outcomes for O-linked adducts.

![Diagram of PhO\textsubscript{G} adduct in DNA opposite natural cytosine in the B conformation and mispaired with guanine adopting a W conformation. Reproduced with permission from reference 88.](image)

**Figure 2 - 9: Depiction of PhO\textsubscript{G} adduct in DNA opposite natural cytosine in the B conformation and mispaired with guanine adopting a W conformation. Reproduced with permission from reference 88.**

Structure-activity relationships have demonstrated that all of the potent aromatic amine carcinogens contain polycyclic structures, while none of the aniline derivatives are potent carcinogens. Carcinogenic potency also follows the para principle, indicating that the most active congeners in extended aromatic structures (biphenyl, fluorene) have amino groups in the para positions. Based on this early data on the simplest phenolic O-linked adduct and behavioral knowledge of corresponding N-linked adducts, it was
desirable to extend the scope of the O-linked study to analyze the effects of ring size, planarity, and chlorine substitution on the behavior of these molecules in a DNA environment.

Recently the Sturla laboratory developed methods for large-scale synthesis of phenolic O-linked C8-dG adducts. Using synthetic methods that were optimized by Mike Kuska of the Manderville laboratory, it allowed the preparation and incorporation of a family of C8-dG adducts (2-7, Scheme 2-1) into DNA. We compared the structural influence of PhO G at G3 of NarI to O-linked C8-dG adducts derived from 4-hydroxybiphenyl (PhPhO dG), 2-naphthol (NaphO dG), 2,4,6-trichlorophenol (TCPO dG), and pentachlorophenol (PCPO dG). These adducts provide an interesting comparison to PhO dG as NaphO dG and PhPhO dG, each contain an additional phenyl ring attached to the C8-phenoxy substituent of PhO dG. For PhPhO dG, the attached phenyl ring is free to rotate while for NaphO dG, the fused ring system possesses the same degree of rotational freedom as the parent PhO dG adduct. The phenols chosen for study are the primary P450 metabolites, of biphenyl and naphthalene. Biphenyl is the unsubstituted derivative of carcinogenic PCBs, and is genotoxic. The National Toxicology Program (NTP) established that naphthalene was carcinogenic to both mice and rats following life-time inhalation exposures, and it is classified as a possible human carcinogen by IARC.

CNBPO dG was also synthesized, in an effort to utilize fluorescent properties gained from cyano group addition, to expand structural results from Tm and CD experiments on PhPhO dG. TCPO dG and PCPO dG both promoted study on the effects of increased chlorination to adduct behavior and provided structural information for a class of adducts that have shown in vitro evidence of formation by banned environmental pollutants (PCP). Given that phenol toxicity correlates with phenoxy radical production, we proposed that O-linked adducts produced by phenoxy radical intermediates may contribute to the
mutagenicity and carcinogenicity of PAHs, PCBs, PCP, and phenolic toxins. Furthermore, relative mutagenic potency of these adducts might be explained mechanistically by specific structural perturbations. Structural studies using $T_m$ and CD measurements assessed how aryl ring size, shape, planarity, and chlorination affected structural conformations of phenolic O-linked C8-dG adducts within the NarI recognition DNA sequence. These comparisons comprise the first half of Chapter 2.

As mentioned in Chapter 1, a quickly evolving tool in oligonucleotide research is the use of Mass spectrometry (MS). MS is used to sequence DNA, confirm correct oligonucleotide structure after synthesis, elucidate structural modifications, and detect nucleic acid reaction products by tandem experiments. Soft ionization techniques such as electrospray ionization (ESI), permit structural studies of fragile oligonucleotides by forming multiply charged gaseous ions. Tandem MS experiments on these ions gather secondary information on gas phase reactivity by collision induced dissociation (CID) which creates distinct fragmentation spectra. Defined fragmentation patterns are often used toxicologically to detect biomarkers in a variety of biological media.

Biomarkers for exposure to toxins are measured in body fluids and tissues and are divided into two categories; markers of internal dose, which indicate the occurrence and extent of exposure to the organism, and markers of effective dose, which measure exposure to the target molecule. Effective dose biomarkers are of greater toxicological significance, because they reflect toxicokinetic parameters of the compound and indicate the extent of DNA damage repair. Good biomarkers are easily measurable, stable, and present at concentrations that reflect exposure levels of the parent compound. Modified nucleosides are widely used as biomarkers for effective dose exposures to various carcinogens and serve as important indicators for disease progression. MS techniques are a sensitive method for biomarker analysis through the input of specific
CID pathways.\textsuperscript{103,104} The use of selective reaction monitoring (SRM) is highly effective for biomarker detection in complex samples because, knowledge of specific fragmentation pathways can produce femtomolar detection limits.\textsuperscript{105} MS has become the method of choice for biomarker detection, and this degree of sensitivity is necessary due to the low incidence rates of some covalent lesions.\textsuperscript{105} MS has also been widely used for detection of polychlorinated phenols in biological and environmental media, and these molecules possess unique dissociation patterns that allow for their unequivocal identification at nanomolar concentrations.\textsuperscript{106,107}

The latter half of Chapter 2 reports on results from various MS experiments on modified O-linked nucleosides from polychlorinated phenols. Analysis of nucleoside adducts, using both positive and negative ionization ESI-MS, explored CID of modified nucleobases in MS\textsuperscript{3} to map gas phase reactivity for the O-linked family of adducts. It was discovered that they produce a common fragment, resulting from homolytic fission of the ether bond, to afford phenyl radicals. These experimental findings prompted synthesis of the corresponding C8-benzyloxy-dG adduct (\textsuperscript{BnO}dG) for comparison, as benzyl radicals are very stable due to resonance. Further work incorporated both \textsuperscript{PCPO}G and \textsuperscript{BnO}G into the 12-mer NarI oligonucleotide (Figure 2-17c) to determine how these adducts undergo CID within DNA. Density functional theory calculations were used to provide a rationale for the relative abundance of the m/z 281 peak, which was influenced by the degree of chlorination of the phenyl ring. The ESI-MS CID patterns discovered for O-linked C8-dG adducts provide a sensitive and reproducible MS detection strategy for effective dose biomarkers following exposure to polychlorinated POPs.
2.2. Experimental Procedures

2.2.1. General methods

A detailed description of the experimental procedures performed in Chapter 2 are given in Appendix A. Specific details of pertinent procedures are described below.

2.2.2. Synthesis of modified O-linked Phosphoramidites

The synthetic strategy used to prepare the modified phosphoramidites is outlined in Scheme 2-1 and follows the protocols provided in detail for the synthesis of the corresponding phosphoramidite of \(^{\text{PhO}dG}\). Synthetic details are outlined briefly below. The compound, 8-benzyloxy-2'-deoxyguanosine \(^{\text{BnO}dG, \text{7}}\), was synthesized according to literature procedures and also described below. The synthesis of 8-bromo-3',5'-O-bis(tert-butyldimethylsilyl)-O\(^{6}\)-(trimethylsilylethyl)-2'-deoxyguanosine \(^{\text{1, Scheme 2-1}}\) was performed as outlined previously. NMR spectra of synthetic samples were recorded at 600 MHz on a Bruker (Billerica, MA) spectrometer in acetone-\(d_6\), DMSO-\(d_6\) or CDCl\(_3\). High-resolution mass spectrometry was conducted at the Biological Mass Spectrometry Center (Guelph, Ontario).

2.2.2.1. Synthesis of O-linked 8-dG Adducts

A general method for the synthesis of O-linked 8-dG adducts has been developed by Dahlmann and Sturla and modified by Kuska and colleagues. As outlined in Scheme 2-1, the synthesis involves base-promoted reactions of phenols with the silyl protected 8-Br-dG analogue \(^{\text{1}}\) in xylene at 135 °C, followed by TBAF-mediated desilylation of O6 and the 3'- and 5'-hydroxyl groups. The reaction, which is a nucleophilic displacement of Br- by phenolate in xylene, was then followed by TBAF
treatment for removal of all protecting groups in a single step. This procedure was used for the preparation of PhPhO\textsubscript{dG}, Nap\textsubscript{dG}, CNBPO\textsubscript{dG}, TCPO\textsubscript{dG}, and PCPO\textsubscript{dG}. Compound 1 was added to a dry round bottom flask and co-evaporated 3X with anhydrous THF to ensure dryness. Tribasic potassium phosphate (K\textsubscript{3}PO\textsubscript{4}, 2 equiv) and phenol (5 equiv) were then added under argon. Rigorously dried, freshly distilled anhydrous xylenes were added and the reaction was stirred at 130 °C for at least 17 h. The reaction was diluted with ethyl acetate and washed 2X with sodium bicarbonate and 2X with water. The solvent was removed in vaccuo and the crude product was isolated by flash chromatography; (90:10) (hexanes: ethyl acetate). The crude material was dissolved in dry THF and 4 equiv of tetrabutylammonium fluoride (TBAF) were added to ensure its final concentration of 0.5 M. The solution was stirred at room temperature for 12 h, THF was removed, and the final product was purified by flash chromatography (92.5: 7.5) (methylene chloride: methanol).

8-(2-naphthoxy)-2'-deoxyguanosine \((\text{NapO}\textsubscript{dG})\) (2). 8-(2-naphthoxy)-2'-deoxyguanosine \((\text{NapO}\textsubscript{dG})\) was synthesized from 1 (2 g, 3.5 mmol), 2-naphthol (2 g, 14.1 mmol), K\textsubscript{3}PO\textsubscript{4} (1.5 g, 7 mmol), and tetrabutylammonium fluoride (TBAF, 4.2 g, 14 mmol) to yield 1.0 g (72% over two steps, isolated as a TBAF salt): \textsuperscript{1}H NMR (600 MHz, DMSO-d\textsubscript{6}) \(\delta\) 8.06-7.83 (m, 4H), 7.54-7.47 (m, 3H), 6.68 (bs, 2H), 6.24 (t, \(J = 6.6\) Hz, 1H), 5.24 (m, 1H), 5.33 (s, 1H), 4.36 (m, 1H), 3.79 (m, 1H), 3.54 (m, 1H), 3.46 (m, 1H), 2.97 (m, 1H), 2.15 (m, 1H); \textsuperscript{13}C NMR (151 MHz, DMSO-d\textsubscript{6}) \(\delta\) 158.9, 156.9, 155.9, 151.9, 150.5, 149.9, 133.5, 131.9, 128.5, 126.9, 119.3, 116.9, 115.7, 89.0, 85.0, 73.8, 64.0, 42.0, 39.2, 32.4; HRMS (ESI) calc for C\textsubscript{20}H\textsubscript{19}N\textsubscript{5}O\textsubscript{5} 410.1459, found 410.1467 (MH\textsuperscript{+}).

8-(4-phenylphenoxy)-2'-deoxyguanosine \((\text{PhPhO}\textsubscript{dG})\) (3). 8-(4-phenylphenoxy)-2'-deoxyguanosine \((\text{PhPhO}\textsubscript{dG})\) was synthesized from 1 (0.5 g, 0.74 mmol), 4-hydroxybiphenyl (0.5 g, 3.0 mmol), K\textsubscript{3}PO\textsubscript{4} (0.3 g, 1.5 mmol), and TBAF (0.9 g, 3 mmol)
to yield 0.3 g (54% over two steps, isolated as a TBAF salt): $^1$H NMR (600 MHz, DMSO-
$_d^6$) δ 10.73 (s, 1H), 7.67 (m, 2H), 7.67 (m, 2H), 7.47 (m, 2H), 7.41 (m, 2H), 7.37 (m, 1H),
6.50 (s, 2H), 6.23 (t, $J = 6.6$ Hz, 1H), 5.28 (s, 1H), 4.85 (s, 1H), 4.36 (s, 1H), 3.78 (m, 1H),
3.45 (m, 2H), 2.93 (m, 1H), 2.17 (m, 1H); $^{13}$C NMR (151 MHz, DMSO-
$_d^6$) δ 155.7, 153.3, 152.9, 149.8, 148.9, 139.3, 137.1, 132.0, 131.4, 128.9, 128.7, 128.6, 127.9,
127.3, 126.6, 120.2, 110.6, 87.3, 81.8, 70.8, 61.9, 36.6; HRMS (ESI) calc for C$_{22}$H$_{21}$N$_5$O$_5$
436.1613, found 436.1616 (MH$^+$).

8-(4-cyanophenylphenoxy)-2'-deoxyguanosine ($^{CNBPO}$dG) (4). 8-(4-
cyanophenylphenoxy)-2'-deoxyguanosine ($^{CNBPO}$dG) was synthesized from 1 (1.0 g, 1.76
mmol), 4-cyanophenyl phenol (1.4 g, 7.0 mmol), K$_3$PO$_4$ (1.0 g, 3.5 mmol), and TBAF (1.2
g, 7 mmol) to yield 0.450 g (54% over two steps, isolated as a TBAF salt): $^1$H NMR (600
MHz, DMSO-
$_d^6$) δ 10.73 (bs, 1H), 7.92 (m, 2H), 7.90 (m, 2H), 7.84 (m, 2H), 7.47 (m, 2H), 7.41, (m, 1H), 6.50 (s, 2H), 6.23 (t, $J = 6.1$ Hz, 1H), 5.28 (s, 1H), 4.85 (s, 1H), 4.36
(s, 1H), 3.78 (m, 1H), 3.45 (m, 2H), 2.93 (m, 1H), 2.17 (m, 1H); $^{13}$C NMR (151 MHz, DMSO-
$_d^6$) δ 155.8, 154.0, 153.5, 149.9, 148.7, 143.7, 135.4, 132.9, 128.6, 127.6, 120.4,
118.9, 110.8, 110.0, 87.4, 81.8, 70.8, 62.0, 57.4, 36.7, 23.0, 19.2, 13.5; HRMS (ESI) calc for C$_{22}$H$_{21}$N$_5$O$_5$
436.1613, found 436.1616 (MH$^+$).

8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine ($^{TCPO}$dG) (5). 8-(2,4,6-
trichlorophenoxy)-2'-deoxyguanosine ($^{TCPO}$dG) was synthesized from 1 (1.0 g, 1.76
mmol), K$_3$PO$_4$ (0.740 g, 3.5 mmol), 2,4,6-trichlorophenol (1.8 g, 8.78 mmol) and TBAF (1.2 g, 1 mmol) to afford 0.53 g (65%) of the final product as a pale yellow powder; $^1$H NMR (300 MHz, DMSO-
$_d^6$) δ 10.63 (s, 1H), 7.89 (s, 2H), 6.30 (s, 2H), 6.09 (m, 1H), 5.29 (m, 1H), 4.86 (m, 1H), 4.22 (m, 1H), 3.81 (m, 1H), 3.50 (m, 1H), 2.99 (m, 1H), 2.21
(m, 1H); $^{13}$C NMR (151 MHz, DMSO-
$_d^6$) δ 158.3, 155.2, 151.1, 145.8, 133.7, 131.1,
8-(pentachlorophenoxy)-2'-deoxyguanosine (PCPO\textsubscript{dG}) (6). 8-(Pentachlorophenoxy)-2'-deoxyguanosine (PCPO\textsubscript{dG}) was synthesized from 1 (2.0 g, 3.55 mmol), K\textsubscript{3}PO\textsubscript{4} (1.5 g, 7.0 mmol), pentachlorophenol (4.7 g, 17.75 mmol) and TBAF (4.3 g, 14.2 mmol) to afford 0.36 g (23%) of the pure product as an off white solid; \textsuperscript{1}H NMR (600 MHz, DMSO-d\textsubscript{6}) δ 10.79 (s, 1H), 6.56 (s, 2H), 6.26 (t, J = 6.6 Hz, 1H), 5.31 (s, 1H), 4.81 (s, 1H), 4.33 (m, 1H), 3.81 (m, 1H), 3.48 (m, 2H), 2.94 (m, 1H), 2.23 (m, 1H); \textsuperscript{13}C NMR (151 MHz, DMSO-d\textsubscript{6}) δ 155.7, 153.7, 150.4, 146.9, 145.3, 131.5, 130.9, 127.5, 110.4, 87.6, 81.9, 79.2, 71.0, 62.1, 36.5, 13.5; HRMS (ESI) calc for C\textsubscript{13}H\textsubscript{11}Cl\textsubscript{2}N\textsubscript{5}O\textsubscript{5} 529.9351 found 529.9354 (MH\textsuperscript{+}).

8-(benzyloxy)-2'-deoxyguanosine (BnO\textsubscript{dG}) (7). 8-(Benzyloxy)-2'-deoxyguanosine (BnO\textsubscript{dG}) was synthesized following a procedure by Bodepudi et al.\textsuperscript{108} Benzyl alcohol (200 mL) was dried over sodium sulphate and distilled by fractional distillation under vacuum. To the dry benzyl alcohol (25 mL, 241 mmol) was added 700 mg of solid sodium under nitrogen. The sodium was allowed to react with the benzyl alcohol at 60 °C until the solid had disappeared and a homogeneous mixture had resulted. DMSO (75 mL) was added to this solution with stirring. 8-Br-dG (3.5 g, 10.3 mmol) was dissolved in 25 mL DMSO and added to the solution. The solution was stirred under argon at 65 °C for 20 h. The reaction was cooled to room temperature, neutralized with glacial acetic acid and the bulk of the DMSO was removed by vacuum distillation. The crude product was re-suspended in diethyl ether, filtered, and re-suspended in acetone. This solid was washed with water and re-crystallized from methanol to afford 2.3 g (62%) of the pure product as a brown solid; \textsuperscript{1}H NMR (300 MHz, DMSO-d\textsubscript{6}) δ 10.97 (bs, 1H), 7.50-7.34 (m, 5H), 6.47 (s, 2H), 6.08 (t, J = 7.2 Hz, 1H), 5.40 (s, 2H), 5.17 (s, 1H), 4.89 (m, 1H),
4.24 (m, 1H), 3.71 (m, 1H), 3.37 (m, 2H), 2.85 (m, 1H), 2.00 (m, 1H); $^{13}\text{C}$ NMR (100 MHz, DMSO-d$_6$) δ 162.3, 156.5, 153.5, 150.7, 150.1, 135.8, 128.4, 128.1, 110.7, 87.4, 81.6, 71.0, 70.5, 62.1, 36.3; HRMS (ESI) calc for C$_{17}$H$_{15}$N$_5$O$_5$ 374.1456 found 374.1449 (MH$^+$). $^1$H and $^{13}$C NMR matched the values from the literature.$^{108}$

2.2.2.2 DMF protection of O-linked modified nucleosides

Established literature procedures were again followed, and are briefly described here.$^{109}$ Four equivalents of N,N-dimethylformamidyl diethyl acetal were added to 20 mL of freshly distilled, dry DMF containing one equivalent of the O-linked nucleoside. The reaction was stirred for 14 h under argon. DMF was removed by repeated co-evaporations with methanol (5X). The resulting precipitate was filtered and dried to give the pure product. If further purification was necessary, flash chromatography was performed using 10:90 (MeOH:CHCl$_3$).

$N^2$-(Dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine (2a). NaphO$_2$dG (1 g, 2.5 mmol) was treated with N,N-(dimethylformamidyl) diethyl acetal (1.4 mL, 10 mmol) to afford 2a as light yellow powder (0.8 g, 70%); $^1$H NMR (600 MHz, CDCl$_3$) δ 8.41 (s, 1H), 7.85 (s, 1H), 7.85-7.77 (m, 4H), 7.46-7.41 (m, 3H), 6.51 (t, J = 5.4 Hz, 1H), 4.86 (m, 1H), 4.71 (m, 1H), 4.13 (s, 1H), 3.88 (m, 1H), 3.70 (m, 1H), 3.14 (s, 3H), 3.07 (m, 1H), 3.05 (s, 3H), 2.26 (m, 1H); $^{13}$C NMR (151 MHz, CDCl$_3$) δ 158.3, 156.3, 156.2, 151.1, 150.2, 148.5, 133.9, 131.2, 129.8, 127.8, 126.6, 125.5, 119.7, 116.1, 115.9, 88.1, 84.3, 73.1, 63.2, 41.4, 39.5, 35.0; HRMS (ESI) calc for C$_{23}$H$_{24}$N$_6$O$_5$ 465.1887 found 465.1891 (MH$^+$).

$N^2$-(Dimethylformamidyl)-8-(4-phenylphenoxy)-2'-deoxyguanosine (3a). PhPhO$_2$dG (0.3 g, 0.7 mmol) was treated with N,N-(dimethylformamidyl) diethyl acetal (0.6 mL, 3.4 mmol) to afford 3a as a light yellow solid (0.26 g, 77%); $^1$H NMR (400 MHz, DMSO-d$_6$) δ
11.41 (s, 1H), 8.54 (s, 1H), 7.74-7.47 (m, 4H), 7.44-7.37 (m, 5H), 6.32 (t, $J = 7.1$ Hz, 1H), 5.34 (d, $J = 3.2$ Hz, 1H), 4.85 (s, 1H), 4.40 (s, 1H), 3.79 (m, 1H), 3.55 (m, 1H), 3.48 (m, 1H), 3.23 (s, 3H), 3.03 (s, 3H), 2.95 (m, 1H), 2.20 (m, 1H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 156.8, 154.8, 154.6, 150.8, 147.8, 146.4, 137.3, 135.3, 126.9, 126.0, 124.7, 118.4, 112.0, 85.4, 79.9, 68.8, 60.0, 55.4, 38.7, 35.0, 32.6, 21.0, 17.2, 11.5; HRMS (ESI) calc for C$_{25}$H$_{26}$N$_6$O$_4$ 491.2035 found 491.2040 (MH$^+$).

$N^2$-(Dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine (4a).

CNBPO$_2$DG (0.35 g, 0.76 mmol) was treated with $N,N$-(dimethylformamidyl) diethyl acetal (0.6 mL, 3.4 mmol) to afford 4a as an off white solid (0.33 g, 83%); $^1$H NMR (600 MHz, CDCl$_3$) δ 8.45 (s, 1H), 7.99 (s, 1H), 7.69 (d, $J = 9$ Hz, 2H), 7.62 (d, $J = 8.4$ Hz, 2H), 7.55 (t, $J = 6.6$ Hz, 2H), 7.51 (t, $J = 2.4$ Hz, 2H), 6.48 (t, $J = 5.4$ Hz, 1H), 4.74 (d, $J = 5.4$ Hz, 1H), 4.16 (s, 1H), 3.88 (m, 1H), 3.72 (m, 1H), 3.16 (s, 3H), 3.10 (m, 1H), 3.05 (s, 3H), 2.28 (m, 1H); $^{13}$C NMR (151 MHz, CDCl$_3$) δ 157.1, 155.8, 155.4, 152.7, 148.9, 147.4, 143.8, 135.2, 131.6, 127.6, 126.6, 119.5, 117.9, 114.8, 109.9, 87.4, 83.2, 72.2, 62.3, 57.8, 40.3, 38.4, 34.0, 28.7, 23.1, 18.7, 12.7; HRMS (ESI) calc for C$_{26}$H$_{25}$N$_7$O$_5$ 516.1987 found 516.1995 (MH$^+$).

$N^2$-(Dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine (5a).

TCPO$_2$DG (0.3 g, 0.64 mmol) was treated with $N,N$-(dimethylformamidyl) diethyl acetal (0.6 mL, 3.4 mmol) to afford 5a as a light yellow powder (0.28 g, 85%); $^1$H NMR (600 MHz, DMSO-d$_6$) δ 8.52 (s, 1H), 7.93 (s, 2H), 6.43 (t, $J = 6$ Hz, 1H), 5.35 (m, 1H), 5.04 (m, 1H), 4.47 (m, 2H), 3.86 (m, 1H), 3.53 (m, 2H), 3.10 (s, 3H), 3.00 (m, 4H), 2.24 (m, 1H); $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 162.6, 159.8, 159.5, 154.3, 151.9, 145.4, 133.4, 130.7, 130.4, 113.3, 89.4, 84.0, 72.6, 65.2, 63.7, 41.9, 38.4, 35.9, 18.8; HRMS (ESI) calc for C$_{19}$H$_{19}$Cl$_3$N$_6$O$_5$ 517.0553 found 517.0568 (MH$^+$).
**N^2-(Dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine (6a).** \( \text{PCPO}_{\text{dG}} \) (0.40 g, 0.76 mmol) was treated with N,N-dimethylformamidyl diethyl acetal (0.68 mL, 3.78 mmol, 5 equiv) to afford 6a as a light yellow powder. Yield (0.34 mg, 78%); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 11.48 (bs, 1H), 8.53 (s, 1H), 6.37 (t, \( J = 6.6 \) Hz, 1H), 5.36 (s, 1H), 4.81 (s, 1H), 4.39 (m, 1H), 3.82 (m, 1H), 3.53 (m, 2H), 3.15 (s, 3H), 3.02 (s, 3H), 2.97 (m, 1H), 2.26 (m, 1H); \(^13\)C NMR (151 MHz, DMSO-\(d_6\)) \( \delta \) 158.1, 157.3, 156.6, 149.0, 147.7, 145.3, 131.5, 131.0, 127.5, 113.7, 87.7, 82.1, 70.9, 62.1, 40.7, 36.9, 34.7, 13.5; HRMS (ESI) calc for C\(_{19}\)H\(_{17}\)Cl\(_5\)N\(_6\)O\(_5\) 584.9773 found 584.9776 (MH\(^+\)).

**N^2-(Dimethylformamidyl)-8-(benzyloxy)-2'-deoxyguanosine (7a).** \( \text{BN}\text{O}_{\text{dG}} \) (1.4 g, 3.75 mmol) was treated with N,N-dimethylformamidyl diethyl acetal (2.74 mL, 16 mmol, 4 equiv) to afford 7a as a light brown solid. Yield (1.10 g, 68%); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 11.32 (1H, s), 8.46 (s, 1H), 7.47 (m, 2H), 7.40 (m, 3H), 6.15 (t, \( J = 6.6 \) Hz, 1H), 5.42 (dd, \( J = 12 \) Hz, 2H), 5.22 (m, 1H), 4.76 (t, \( J = 4.8 \) Hz, 1H), 4.27 (m, 1H), 3.71 (m, 1H), 3.46 (m, 1H), 3.38 (m, 1H), 3.11 (s, 3H), 2.99 (s, 3H), 2.87 (m, 1H), 2.05 (m, 1H); \(^13\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \) 157.7, 156.6, 156.4, 151.5, 148.6, 135.8, 128.5, 128.3, 128.2, 114.1, 87.4, 81.6, 70.9, 70.7, 62.1, 40.6, 36.7, 34.6; HRMS (ESI) calc for C\(_{20}\)H\(_{24}\)N\(_6\)O\(_5\) 429.1886 found 429.1877 (MH\(^+\)).

### 2.2.2.3 DMT protection of DMF-protected O-linked nucleosides

Dimethoxytrityl protection of the 5'OH group was employed on the DMF protected O-linked nucleosides, according to established literature procedures,\(^{110}\) with some modifications briefly described here. 4,4'-Dimethoxytrityl chloride (DMTCl, 1.2 equiv), was dissolved in 10 mL anhydrous pyridine in a dropping funnel under nitrogen, and slowly added over 40 min to one equivalent of \( N^2-(\text{Dimethylformamidyl}) \) protected O-linked nucleoside in freshly distilled DMF. The reaction vessel was purged 3X with argon.
before addition. The reaction was stirred at room temperature for 4 h, diluted with ethyl acetate, washed 2X with brine, and 2X with water. The organic layer was dried with sodium sulphate and evaporated to dryness. The residue was dissolved in 2 mL of methylene chloride and 20 mL of hexanes was added. A precipitate formed and the suspension was stirred for 14 h at room temperature. The suspension was filtered, and the crude product was purified by flash chromatography (5:5:90 methanol: triethylamine: methylene chloride).

5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-N²-(dimethylformamidyl)-8-(2-naphthoxy)-2ꞌ-deoxyguanosine (2b). Compound 2a (0.8 g, 1.7 mmol) was treated with DMT-Cl (0.9 g, 2.6 mmol) in dry pyridine to afford 2b as an off-white solid (1 g, 76%): ¹H NMR (600 MHz, CDCl₃) δ 9.03 (bs, 1H), 8.47 (s, 1H), 7.79 (m, 1H), 7.67 (m, 2H), 7.59 (m, 1H), 7.42 (m, 2H), 7.29 (m, 2H), 7.16-7.07 (m, 8H), 6.61 (m, 4H), 6.48 (t, J = 6.9 Hz, 1H), 4.67 (m, 1H), 4.02 (m, 1H), 3.64 (s, 6H), 3.35 (m, 1H), 3.21 (m, 1H), 3.02 (s, 3H), 3.00 (m, 1H), 2.98 (s, 3H), 2.53 (m, 1H), 2.36 (m, 1H); ¹³C NMR (151 MHz, CDCl₃) δ 158.5, 157.9, 157.1, 156.1, 150.7, 149.2, 144.7, 135.8, 133.9, 131.3, 130.0, 129.6, 128.2, 127.7, 126.9, 126.6, 125.5, 119.9, 116.5, 114.8, 113.0, 86.4, 85.3, 81.9, 73.2, 64.4, 55.2, 46.2, 41.3, 37.5, 35.2; HRMS (ESI) calc for C₄₄H₄₂N₆O₇ 767.3185, found 767.3196 (MH⁺).

5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-N²-(dimethylformamidyl)-8-(4-phenylphenoxy)-2ꞌ-deoxyguanosine (3b). Compound 3a (0.26 g, 0.53 mmol) was treated with DMT-Cl (0.2 g, 0.6 mmol) in dry pyridine to afford 3b as an off-white solid (0.2 g, 54%); ¹H NMR (600 MHz, CDCl₃) δ 9.29 (bs, 1H), 8.50 (s, 1H), 7.52 (m, 2H), 7.43 (m, 4H), 7.31 (m, 4H), 7.25-7.12 (m, 10H), 6.75 (m, 4H), 6.43 (t, J = 6.6 Hz, 1H), 5.27 (m, 1H), 4.66 (m, 1H), 4.06 (m, 1H), 3.66 (s, 6H), 3.42 (m, 1H), 3.37 (m, 1H), 3.00 (s, 3H), 2.97 (s, 3H), 2.86 (m, 1H), 2.37 (m, 1H); ¹³C NMR (151 MHz, CDCl₃) δ 158.5, 158.0, 157.3, 156.1, 152.6, 150.8, 149.2, 144.7, 140.4, 138.3, 135.8, 130.0, 128.8, 128.3, 127.8, 127.1, 120.3,
114.7, 113.1, 86.3, 85.3, 81.8, 73.1, 64.5, 55.2, 45.9, 41.3, 37.5, 35.2; HRMS (ESI) calc for C_{46}H_{44}N_{6}O_{7} 793.3341 found 793.3346 (MH^+).

5'-O-(4,4'-Dimethoxytrityl)-N^2-(dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine (4b). Compound 4a (0.3 g, 0.58 mmol) was treated with DMT-Cl (0.3 g, 0.87 mmol) in dry pyridine to afford 4b as an off-white solid (0.31 g, 74%); ^1H NMR (600 MHz, CDCl_3) δ 9.36 (s, 1H), 8.49 (s, 1H), 7.71 (d, J = 7.8 Hz, 2H), 7.62 (d, J = 7.8 Hz, 2H), 7.45 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 7.8 Hz, 2H), 7.23-7.14 (mm, 9H), 6.70 (d, J = 7.8 Hz, 4H), 6.49 (t, J = 6.6 Hz, 1H), 4.68 (m, 1H), 4.08 (m, 1H), 3.70 (s, 6H), 3.38 (m, 1H), 3.25 (m, 1H), 3.05 (s, 3H), 3.01 (s, 3H), 2.98 (m, 1H), 2.39 (m, 1H); ^13C NMR (151 MHz, CDCl_3) δ 158.5, 157.9, 157.3, 156.2, 153.6, 150.4, 149.2, 144.8, 144.7, 136.1, 135.8, 135.7, 132.7, 130.0, 129.2, 128.4, 128.1, 127.9, 127.8, 127.6, 126.9, 126.6, 119.0, 114.6, 113.2, 113.1, 110.9, 86.3, 85.3, 81.8, 73.0, 64.4, 55.2, 41.4, 37.5, 35.2; HRMS (ESI) calc for C_{47}H_{45}N_{7}O_{7} 818.3298, found 818.3305 (MH^+).

5'-O-(4,4'-Dimethoxytrityl)-N^2-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine (5b). Compound 5a (0.4 g, 0.76 mmol) was treated with DMT-Cl (0.4 g, 1.2 mmol) to afford 5b as a light yellow powder. Yield (0.6 g, 87%); ^1H NMR (600 MHz, Acetone-d_6) δ 8.58 (s, 1H), 7.67 (s, 2H), 7.44 (m, 2H), 7.38 (m, 4H), 7.25 (m, 2H), 7.16 (m, 1H), 6.71 (m, 4H), 6.64 (t, J = 7.2 Hz, 1H), 4.67 (m, 1H), 4.51 (m, 2H), 4.21 (m, 1H), 3.73 (s, 6H), 3.46 (m, 1H), 3.23 (m, 1H), 3.22 (m, 1H), 3.11 (s, 3H), 3.06 (s, 3H), 2.37 (m, 1H); ^13C NMR (151 MHz, Acetone-d_6) δ 163.7, 161.1, 160.8, 160.7, 160.1, 155.8, 153.3, 147.6, 146.8, 138.3, 138.2, 134.4, 132.3, 132.2, 131.9, 131.3, 130.3, 129.7, 128.7, 115.0, 114.7, 88.6, 88.0, 84.6, 66.7, 65.9, 56.8, 42.2, 36.3, 19.6; HRMS (ESI) calc for C_{40}H_{37}Cl_{3}N_{6}O_{7} 819.1859 found 819.1866 (MH^+).
5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-N²-(dimethylformamidyl)-8-(pentachlorophenoxy)-2ꞌ-deoxyguanosine (6b). Compound 6a (0.34 g, 0.58 mmol) was treated with DMTCl (0.23 g, 0.87 mmol), to afford 6b as a light yellow powder. Yield (0.45 g, 86%); ¹H NMR (600 MHz, CDCl₃) δ 8.54 (s, 1H), 7.32-7.10 (m, 9H), 6.69 (m, 4H), 6.52 (t, J = 7.2 Hz, 1H), 4.66 (m, 1H), 4.11 (m, 1H), 3.72 (s, 6H), 3.50 (m, 1H), 3.20 (m, 2H), 3.12 (s, 3H), 3.05 (s, 3H), 3.00 (m, 1H), 2.41 (m, 1H); ¹³C NMR (151 MHz, CDCl₃) δ 157.9, 156.9, 156.4, 149.6, 148.4, 144.9, 144.4, 135.6, 135.5, 132.0, 129.9, 129.2, 128.0, 127.9, 127.8, 126.9, 114.5, 113.2, 113.0, 112.9, 86.5, 85.2, 81.9, 73.6, 64.1, 55.3, 41.4, 37.2, 35.2, 34.6; HRMS (ESI) calc for C₄₀H₃₅Cl₅N₆O₈ 887.1080 found 887.1076 (MH⁺).

5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-N²-(dimethylformamidyl)-8-(benzyl oxy)-2ꞌ-deoxyguanosine (7b). Compound 7a (1.0 g, 2.34 mmol) was treated with DMTCl (1.2 g, 3.54 mmol) to afford 7b as an off-white solid. Yield (0.70 g, 96%); ¹H NMR (600 MHz, DMSO-d₆) δ 11.32 (s, 1H), 8.44 (s, 1H), 7.30 (m, 5H), 7.17 (m, 9H), 6.77 (m, 4H), 6.27 (t, J = 6 Hz, 1H), 5.36 (d, J = 12 Hz, 1H), 5.26 (d, J = 4.8 Hz, 1H), 5.19 (d, J = 12 Hz, 1H), 4.23 (m, 1H), 3.84 (m, 1H), 3.72 (s, 6H), 3.10 (m, 1H), 3.08 (s, 3H), 3.01 (s, 3H), 2.96 (m, 1H), 2.83 (m, 1H), 2.15 (m, 1H); ¹³C NMR (151 MHz, DMSO-d₆) δ 158.0, 157.9, 157.6, 156.7, 156.2, 151.5, 149.5, 144.9, 135.6, 135.5, 129.6, 128.4, 128.3, 128.0, 127.7, 127.6, 126.5, 113.9, 113.0, 112.9, 85.4, 85.2, 81.2, 70.8, 70.6, 64.4, 55.0, 54.9, 40.7, 40.6, 37.1, 34.6; HRMS (ESI) calc for C₄₁H₄₂N₆O₇ 731.3193 found 731.3202 (MH⁺).

2.2.2.4 Phosphitylation of DMF-DMT protected O-linked nucleosides

Phosphitylation reactions were carried out as described in the literature, with modifications described here. 5ꞌ-O-(4,4ꞌ-dimethoxytrityl)-N²-(dimethylformamidyl)-8-O-linked nucleosides were co-evaporated 3X from freshly distilled, dry THF. The
reaction vessel was purged with argon and vacuum 3X. Freshly degassed, dry methylene chloride (6 mL), was added and the starting material dissolved completely to form a yellow solution. Dry triethylamine (TEA, 4 equiv) and 1.5 equiv of 2-cyanoethyl N,N-diisopropylchlorophosphoramidite were added under argon. The solution was stirred at room temperature for 30 min while monitoring the completion of the reaction by TLC. The solution was added dropwise into a degassed sodium bicarbonate solution and washed 3X. The organic layer was removed, and the majority of the methylene chloride was evaporated off. The product was re-crystallized out of cold (-78 °C) hexanes to give an off white foamy solid. The crude product was purified further by column chromatography (5:5:90 methanol:triethylamine:methylene chloride) to afford the pure product as a foamy solid.

3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N^2-(dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine (2c). Compound 2b (0.5 g, 0.65 mmol) was treated with 2-cyanoethyl-N,N-diisopropyl-chloro-phosphoramidite (0.22 mL, 0.98 mmol) in dry dichloromethane in the presence of triethylamine to afford 2c as a white foam (0.4 g, 63%); 1H NMR (600 MHz, Acetone-d₆) δ 10.15 (s, 1H), 8.60 (s, 1H), 7.93 (m, 1H), 7.90-7.87 (m, 1H), 7.82-7.80 (m, 1H), 7.73 (m, 1H), 7.54-7.48 (m, 2H), 7.41-7.37 (m, 2H), 7.35 (m, 1H), 7.26-7.11 (m, 7H), 6.72-6.66 (m, 4H), 6.56 (m, 1H), 5.02-4.88 (m, 1H), 4.20 (m, 1H), 4.11 (m, 1H), 3.89-3.79 (m, 1H), 3.70 (s, 6H), 3.67-3.57 (m, 3H), 3.59-3.47 (m, 1H), 3.33 (m, 2H), 3.25 (m, 2H), 3.17 (s, 3H), 3.07 (s, 3H), 2.72 (m, 1H), 2.64-2.51 (m, 2H), 1.18-1.13 (m, 9H), 1.04 (m, 3H); 31P NMR (121.4 MHz, Acetone-d₆) δ 149.06, 148.76; HRMS (ESI) calc for C₅₅H₅₉N₈O₈P 967.4273 found 967.4276 (MH⁺).
3′-O-[(2-Cyanoethyl)(diisopropylamino)phosphino]-5′-O-(4,4′-dimethoxytrityl)-N2-(dimethylformamidyl)-8-(4-phenylphenoxy)-2′-deoxyguanosine (3c). Compound 3b (0.23 g, 0.29 mmol) was treated with 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite (0.1 mL, 0.44 mmol) in dry dichloromethane in the presence of triethylamine to afford 3c as a white foam (0.14 g, 48%); 1H NMR (600 MHz, Acetone-d6) δ 8.59 (s, 1H), 7.66 (m, 2H), 7.61 (m, 2H), 7.46 (m, 2H), 7.38 (m, 1H), 7.34 (m, 2H), 7.26-7.17 (mm, 10H), 6.75-6.70 (m, 4H), 6.51 (t, J = 6.2 Hz, 1H), 4.97-4.84 (m, 1H), 4.17 (m, 1H), 3.78-3.90 (m, 2H), 3.70 (s, 6H), 3.62 (m, 3H), 3.32 (m, 1H), 3.24 (m, 2H), 3.17 (s, 3H), 3.10 (s, 3H), 2.47-2.61 (m, 2H), 1.15 (m, 8H), 1.05 (m, 4H); 31P NMR (121.4 MHz, Acetone-d6) δ 148.54; HRMS (ESI) calc for C55H61N8O8P 993.4430 found 993.4432 (MH+).

3′-O-[(2-Cyanoethyl)(diisopropylamino)phosphino]-5′-O-(4,4′-dimethoxytrityl)-N2-(dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2′-deoxyguanosine (4c). Compound 4b (0.31 g, 0.38 mmol) was treated with 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite (0.13 mL, 0.57 mmol) in dry dichloromethane in the presence of triethylamine to afford 4c as a faint brown foam (0.28 g, 74%); 1H NMR (300 MHz, CDCl3) δ 8.89 (s, 1H), 8.51 (s, 1H), 7.69 (m, 2H), 7.61 (m, 2H), 7.44 (m, 2H), 7.40 (m, 2H), 7.25-7.11 (mm, 9H), 6.67 (m, 4H), 6.46 (t, J = 6.6 Hz, 1H), 4.78 (m, 1H), 4.12 (m, 1H), 3.78 (m, 1H), 3.68 (s, 6H), 3.54-3.42 (m, 7H), 3.28 (m, 2H), 3.05 (s, 3H), 3.01 (s, 3H), 2.72 (m, 4H), 2.54 (m, 1H), 2.39 (m, 1H), 1.13 (m, 9H), 1.05 (m, 3H); 31P NMR (121.4 MHz, CDCl3) δ 149.09, 148.79; HRMS (ESI) calc for C56H60N9O8P 1002.4423 found 1002.4416 (MH+).

3′-O-[(2-Cyanoethyl)(diisopropylamino)phosphino]-5′-O-(4,4′-dimethoxytrityl)-N2-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2′-deoxyguanosine (5c). Compound 5b (0.4 g, 0.5 mmol) was treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.3 mL, 2.0 mmol) in the presence of triethylamine to
afford 5c as a foamy light yellow solid (0.4 g, 82%); \(^1\)H NMR (600 MHz, Acetone-d\(_6\)) δ 8.61 (s, 1H), 7.64 (s, 2H), 7.41 (m, 3H), 7.22-7.11 (m, 5H), 6.77-6.70 (m, 6H), 6.55 (m, 1H), 4.93-4.83 (mm, 1H), 4.26 (m, 1H), 4.21-4.10 (mm, 2H), 3.75 (m, 7H), 3.66 (m, 4H), 3.51 (m, 2H), 3.31 (m, 2H), 3.19 (s, 3H), 3.10 (s, 3H), 2.76 (m, 2H), 2.65 (m, 1H), 1.63-1.18 (m, 6H), 1.02 (m, 6H); \(^{31}\)P NMR (121.4 MHz, Acetone-d\(_6\)) δ 149.47, 149.01; HRMS (ESI) calc for C\(_{49}\)H\(_{54}\)Cl\(_3\)N\(_8\)O\(_7\)P 1003.2999 found 1003.2992 (MH\(^+\)).

3ꞌ-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5ꞌ-O-(4,4ꞌ-dimethoxytrityl)-\(N^2\)-(dimethylformamidyl)-8-(pentachlorophenoxy)-2ꞌ-deoxyguanosine (6c). Compound 6b (0.45 g, 0.50 mmol) was treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.18 mL, 0.76 mmol) in the presence of triethylamine (0.31 mL, 3.02 mmol) to yield 6c as a pale yellow foam (0.42 g, 77%); \(^1\)H NMR (300 MHz, CD\(_3\)CN) δ 9.52 (bs, 1H), 8.54 (s, 1H), 7.34 (m, 2H), 7.24-7.16 (m, 7H), 6.74-6.69 (m, 4H), 6.48 (t, \(J = 6.6\) Hz, 1H), 4.78 (m, 1H), 4.21 (m, 1H), 4.10-4.03 (mm, 3H), 3.81 (m, 1H), 3.71 (s, 6H), 3.53 (m, 3H), 3.45 (m, 2H), 3.26 (m, 2H), 3.10 (s, 3H), 3.02 (s, 3H), 2.75 (t, \(J = 6.0\) Hz, 2H), 2.62 (m, 1H), 2.53 (m, 1H), 2.21 (m, 1H), 1.14 (m, 8H), 1.10 (m, 4H); \(^{31}\)P NMR (121 MHz, CD\(_3\)CN) δ 148.42, 148.24; HRMS (ESI) calc for C\(_{48}\)H\(_{52}\)Cl\(_5\)N\(_9\)O\(_7\)P 1071.2257 found 1071.2252 (MH\(^+\)).

3ꞌ-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5ꞌ-O-(4,4ꞌ-dimethoxytrityl)-\(N^2\)-(dimethylformamidyl)-8-(benzyl oxy)-2ꞌ-deoxyguanosine (7c) Compound 7b (0.5 g, 6.85 mmol) was treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.230 mL, 10.3 mmol) in the presence of excess triethylamine (0.38 mL, 2.74 mmol) to afford 7c as a white foam (406 mg, 62%); \(^1\)H NMR (300 MHz, Acetone-d\(_6\)) δ 10.05 (bs, 1H), 8.53 (s, 1H), 7.41-7.17 (mm, 15H), 6.81-6.74 (m, 5H), 6.37 (m, 1H), 5.41 (m, 1H), 5.31 (m, 1H), 4.86-4.67 (m, 1H), 4.14-4.03 (m, 1H), 3.74 (s, 1H), 3.71 (m, 1H), 3.62-3.49 (m, 3H), 3.33-3.26 (m, 1H), 3.14 (s, 1H), 3.12 (m, 1H), 3.05 (s, 1H), 3.69 (m, 1H), 2.53 (m,
1H), 2.43 (m, 1H), 1.45 (m, 1H), 1.27-1.21 (m, 6H), 1.17-1.11 (m, 8H), 1.05-0.99 (m, 3H), 0.87-0.83 (m, 2H); $^{31}$P NMR (121 MHz, CDCl$_3$) δ 148.76, 146.13; HRMS (ESI) calc for C$_{50}$H$_{59}$N$_6$O$_7$P 915.4314 found, 915.4311 (MH$^+$).

2.2.3. Synthesis and purification of O-linked modified NarI Oligonucleotides.

The modified NarI 12mer oligonucleotides were synthesized using the solid phase DNA synthetic method, performed on a 1 µmol scale on a BioAutomation Corp. MerMade 12 automatic synthesizer using the modified phosphoramidites. Recall that NarI is 12 bases long with the sequence 5′-CTCG$_1$G$_2$CG$_3$CCATC-3′. The O-linked modified dG base was inserted into position G$_3$ for all modified sequences. Standard coupling times and reagents were employed for DNA synthesis. The machine was operated by Michael Sproviero in the Manderville laboratory at the University of Guelph. Following synthesis, the modified oligonucleotides were cleaved from the solid support and deprotected using 2 mL of a 30% ammonium hydroxide solution at 55 °C for 12 h. Following filtration with a 0.2 µm PVDF microfilter from Mandel Scientific, the crude oligonucleotides were purified by RP-HPLC as described in section 1.3.3.

2.2.4. DNA Quantification.

Stock solutions of DNA (1.00 mL) were prepared in Milli-Q purified water. The DNA stock (5 µL) was added to 2000 µL of purified water and an absorption scan at 260 nm on a Cary 300-Bio UV–visible spectrophotometer equipped with a Peltier block-heating unit and automated temperature controller. Standard 10 mm light path quartz glass cells from Hellma GmbH & Co. (Concord, ON) were used. Scans were repeated
three times to determine the concentration of the stock solution using Beers law \( A = εc)l \). Molar absorptivities \( ε \) of unmodified NarI were used for the modified NarI strand and were assumed to be a good approximation of \( ε \) for the modified strands. These values were calculated online using Integrated DNA Technologies (IDT) OligoAnalyzer version 3.1.

2.2.5. UV Melting Studies.

UV melting experiments were conducted using a Cary 300-Bio UV–vis spectrophotometer equipped with a 6 × 6 multicell Peltier block-heating unit using Hellma 114-QS 10 mm light path cells. Oligonucleotide duplex samples were prepared in 50 mM Na$_2$PO$_4$ buffer with 100 mM NaCl (pH 7) to a concentration of 6 μM using 1 equiv of complementary strand. The UV absorption at 260 nm was monitored as a function of temperature while heating and cooling between 10 to 90 °C at a rate of 0.5 °C/min, which was repeated five times. Thermal melting temperatures \( T_m \) were determined using hyperchromicity calculations provided in the Varian Thermal software.

2.2.6. Circular Dichroism (CD) Experiments.

Spectra were recorded on a Jasco J-815 CD spectropolarimeter equipped with a 1 × 6 Multicell Block Peltier, a thermal controller, and a Julabo AWC 100 water circulator unit. CD spectra were recorded using 110-QS cells with a light path of 1 mm. Samples of duplex DNA were prepared in 50 mM Na$_2$HPO$_4$ buffer and 100 mM NaCl (pH 7) to a concentration of 6 μM. Spectra were recorded at 15 °C between 200 nm and 400 nm, with a bandwidth of 1 nm at a rate of 50 nm/min. The spectra were the averages of four accumulations that were smoothed using the Jasco software.
2.2.7. Fluorescent Properties of $^{\text{CNBPO}}dG$ Nucleoside

Stock solutions were made in DMSO, due to sparing solubility in other solvents, to a concentration of 4 mM. Spectroscopic solutions of the modified nucleosides were prepared in 10 mM MOPS buffer, pH 7, with 100 mM NaCl, and made to a concentration of 20 $\mu$M for fluorescence measurements. Fluorescence spectra were recorded at the excitation wavelength (absorbance maxima) for $^{\text{CNBPO}}dG$ from 10 nm above the excitation wavelength, to 600 nm. Fluorescence spectra were recorded using quartz cells (101-QS) with a light path of 10 x 10 mm, excitation and emission slit widths were kept constant at 2.5 nm.

2.2.8. Fluorescence Measurements of $^{\text{CNBPO}}G$ Modified NarI Oligonucleotides.

Solutions of NarI modified at G$_3$ with $^{\text{CNBPO}}G$ were made to 6 $\mu$M in 50 mM Na$_2$HPO$_4$ buffer and 100 mM NaCl (pH 7). Complementary strands with $N = (C, G, A, T, THF$ and 10mer) were added at 6 $\mu$M concentration. The strands were annealed by repeated heating and slow cooling. Fluorescent measurements were performed before and after annealing.

2.2.9. Mass Spectrometry.

2.2.9.1 Mass Spectrometry of Oligonucleotides.

MS experiments, confirming correct synthesis of modified oligonucleotides, were conducted on a Bruker AmaZon SL quadrupole ion trap SL spectrometer. Spectral data was acquired in the negative ionization mode using an electrospray ionization source (Bruker Daltronics, Milton, ON). The DNA samples were dissolved in a 90% Milli-Q filtered water, 10% MeOH mixture with 0.1% ammonium acetate, and injected directly into the electrospray source at 20 uL/min. The ionization was conducted using the
following ESI settings: nebulizer gas pressure (40 psi); drying gas flow (10 L/min); drying gas temperature (200 °C); spray voltage, (−4000 V). The mass range was m/z 70–2000, and the scan rate was 8100 m/z/s.

2.2.9.2 Tandem Mass Spectrometry (MS²) of Modified Nucleosides.

MS² studies of the nucleoside adducts were performed using a Bruker AmaZon quadrupole ion trap mass spectrometer in both positive and negative ionization modes. The ESI source was operated at the same settings as those described for the MS analysis of oligonucleotides. In addition, a spray voltage of +4000V was used for positive mode experiments. MS² spectra were obtained by CID with helium gas after isolation of appropriate precursor ions. Collision energies were set to 0.36 V for MS² of positive ions and 0.25 V for MS² of negative ions. Nucleoside stock solutions (6 mM in DMSO) were diluted with milliQ water to obtain a final nucleoside concentration of ~10 µM. Diluted solutions were directly infused into the ESI source at 10 µL/min. To compare the abundance of the fragment peaks for sugar loss versus phenyl radical loss in ESI²-MS² spectra, collision energy was set as a constant, and CID was performed for 1.0 min. The MS² spectra for the monoisotopic parent ion were collected for 1 min, averaged and the absolute peak intensities of the parent ion [M − H]⁻ and the product ions were measured using the AmaZon software. The relative ratios of the fragment ions were calculated by dividing the absolute peak height of the fragment by the sum of all significant ion peak heights in the MS² spectrum.

2.2.10. Computational Details.

The preferred conformations of the adducts Ph-O-dG (8), 4-Cl-O-dG (9), 2,4-DCl-O-dG (11), TCP-O-dG (5) and PCP-O-dG (6) were determined using an internal coordinate Monte Carlo¹¹⁴ conformational search, initially performed using
HyperChem\textsuperscript{115} software suite. Based on these structures, full optimizations (all constraints released) were performed with B3LYP/6-31G(d) to identify the global minimum geometries of the adducts. In the present work, these global minimum structures were used to calculate the bond-dissociation energies (BDEs) associated with the bond connecting the C8 oxygen atom and the (chlorosubstituted or unsubstituted) phenyl moiety of the adducts based on the homolytic cleavage reaction depicted in Scheme 2-4 using B3LYP/6-31G(d). Additionally, the effects of increasing chlorine substitution on the stability of the phenyl radical were studied using the isodesmic reaction between the phenoxy radical and benzene (chlorosubstituted or unsubstituted, Scheme 2-3). The reaction free energies were calculated at the B3LYP/6-31G(d) level. All B3LYP calculations were performed using Gaussian 09, revision C.01. by Purshotam Sharma at the University of Lethbridge, Lethbridge, Alberta.

2.3. Results and Discussion.

2.3.1. Synthesis and Incorporation of O-linked adducts into Oligonucleotides

The strategy employed to synthesize the modified phenolic O-linked nucleosides is outlined in Scheme 2-1 and involved a method originally developed by Dahlmann and Sturla\textsuperscript{91} and modified by Kuska and coworkers.\textsuperscript{77} Bn\textsuperscript{O}dG was synthesized according to well established literature procedures developed by Bodepudi et. al.\textsuperscript{108} Some of the O-linked nucleoside adducts could only be isolated as the TBAF salt despite multiple column chromatography purification steps. This salt did not interfere with the subsequent \(N^\text{F}\)-dimethylformamidyl protection step, and the products from this reaction (2a and 3a) were isolated in satisfactory yield (\(~\text{70}\%\)) and purity without the presence of TBAF.

During my initial attempts at O-linked coupling of chlorinated phenols, extremely
poor yields were observed for $^{31}$P-POdG (23%). Modifications to improve reaction yield included multiple recrystallizations of PCP ensuring reagent purity, rigorously drying all solvents and reagents to minimize nucleophilic competition from water, and extension of reaction time for multiple days. Despite these modifications, the yield remained poor which was attributed to the low nucleophilicity of phenolates with electron withdrawing chlorine atoms. Despite these challenges, the pure product was obtained in sufficient quantity to proceed with subsequent synthetic steps. Standard protection strategies were then employed to convert the family of O-linked nucleosides into the corresponding phosphoramidites 2c-7c. The NMR spectra of all synthetic products are shown in Appendix C. The modified phosphoramidites 2c-7c all contained some oxidized impurity that was difficult to separate from the unoxidized product. This peak appeared between 10-20 ppm on the $^{31}$P NMR of the amidites and is identified on the spectra (Appendix C). Integration was used to calculate the percent impurity in the amidite and concentrations of the phosphoramidite solutions accounted for the decreased mass of pure product used. The phosphoramidite solutions were loaded onto the DNA synthesizer to prepare Narl oligonucleotides with site-specific modification at the G$_3$ position, as shown in Figure 2-19. Purification of modified oligonucleotides was carried out using RP-HPLC on a semi-preparative scale. Confirmation of structure and purity was established using negative electrospray ionization mass spectrometry (ESI-MS) as shown in Appendix B.
Scheme 2 - 1: Synthesis of modified O-linked phosphoramidites (2c-7c).

![Scheme 2 - 1: Synthesis of modified O-linked phosphoramidites (2c-7c).](image)

2.3.2. UV-Thermal Melting Study Results.

Thermal melting temperatures ($T_m$) of the unmodified and modified Nar1 duplexes are given in Table 2-1. For comparison to duplex structures containing $X = \text{PhPh}O\text{G}$, $\text{Naph}O\text{G}$, $\text{TCP}O\text{G}$ and $\text{PCP}O\text{G}$ $T_m$ values obtained previously for $X = \text{Ph}O\text{G}$ are also provided.\textsuperscript{77}
In general, when the complementary strand contained a base opposite the adduct (N = C, G, A or T), the O-linked adducts were destabilizing compared to the unmodified duplexes and showed negative $\Delta T_m$ values. For $X = \text{PhPhO} G$ and $\text{NaphO} G$ compared to $X = \text{PhO} G$ ($\Delta T_m$ values provided in brackets, Table 2-1), the bulkier lesions had very little impact when N = C or G but were stabilizing opposite A ($\Delta T_m \sim 4.5 ^\circ C$). For $X = \text{TcPO} G$ and $\text{PCPO} G$ opposite (N = C, G, A or T), significant destabilization was observed for all opposing bases compared to unmodified duplex. The chlorinated adducts were more destabilizing compared to $\text{PhO} G$ except when N = A, which demonstrated very little impact (Table 2-1). The $\text{PhPhO} G$ adduct was also stabilizing opposite T ($\Delta T_m = 3.3 ^\circ C$), while $\text{NaphO} G$ had no effect. In general, the chlorinated adducts showed a larger destabilizing effect compared to $\text{NaphO} G$ and $\text{PhPhO} G$ opposite the four bases ($\Delta T_m = -6.2 ^\circ C - -17.6 ^\circ C$, Table 2-1).
Table 2 - 1: Thermal Parameters of C8-Phenoxy-G Modified Nal Oligonucleotides

<table>
<thead>
<tr>
<th>X</th>
<th>N</th>
<th>Tm (°C)</th>
<th>ΔTm</th>
<th>X</th>
<th>N</th>
<th>Tm (°C)</th>
<th>ΔTm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhO</td>
<td>G</td>
<td>52.8</td>
<td>10.8</td>
<td>G</td>
<td>T</td>
<td>47.1</td>
<td>6.3</td>
</tr>
<tr>
<td>PhPhO</td>
<td>G</td>
<td>52.5</td>
<td>-11.1 (-0.3)</td>
<td>PhPhO</td>
<td>G</td>
<td>50.4</td>
<td>-3.0 (+3.3)</td>
</tr>
<tr>
<td>NaphO</td>
<td>G</td>
<td>51.8</td>
<td>-11.8 (-1.0)</td>
<td>NaphO</td>
<td>G</td>
<td>47.1</td>
<td>-6.3 (0)</td>
</tr>
<tr>
<td>TCPO</td>
<td>G</td>
<td>46.0</td>
<td>-17.6 (-6.8)</td>
<td>TCPO</td>
<td>G</td>
<td>43.7</td>
<td>-9.5 (-3.2)</td>
</tr>
<tr>
<td>PCPO</td>
<td>G</td>
<td>46.3</td>
<td>-17.3 (-6.5)</td>
<td>PCPO</td>
<td>G</td>
<td>43.7</td>
<td>-9.9 (-3.4)</td>
</tr>
<tr>
<td>G</td>
<td>PhO</td>
<td>54.0</td>
<td>-1.9</td>
<td>G</td>
<td>THF</td>
<td>45.7</td>
<td>-</td>
</tr>
<tr>
<td>PhPhO</td>
<td>G</td>
<td>52.1</td>
<td>-0.8 (+1.1)</td>
<td>PhPhO</td>
<td>G</td>
<td>50.5</td>
<td>+4.8 (+8.2)</td>
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<tr>
<td>NaphO</td>
<td>G</td>
<td>52.9</td>
<td>-1.1 (+0.8)</td>
<td>NaphO</td>
<td>G</td>
<td>49.7</td>
<td>+4.0 (+7.4)</td>
</tr>
<tr>
<td>TCPO</td>
<td>G</td>
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<td>TCPO</td>
<td>G</td>
<td>42.7</td>
<td>-3 (+0.4)</td>
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<tr>
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<td>-8.5 (-6.6)</td>
<td>PCPO</td>
<td>G</td>
<td>46.4</td>
<td>+0.7 (+4.1)</td>
</tr>
<tr>
<td>G</td>
<td>PhO</td>
<td>51.4</td>
<td>-7.0</td>
<td>G</td>
<td>-2</td>
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<td>-</td>
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<tr>
<td>PhPhO</td>
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<td>44.4</td>
<td>-2.4 (+4.6)</td>
<td>PhPhO</td>
<td>G</td>
<td>35.0</td>
<td>-4.4</td>
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<tr>
<td>NaphO</td>
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<td>49.0</td>
<td>-2.9 (+4.1)</td>
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<td>G</td>
<td>46.0</td>
<td>+6.6 (+11.0)</td>
</tr>
<tr>
<td>TCPO</td>
<td>G</td>
<td>44.0</td>
<td>-7.4 (0.4)</td>
<td>TCPO</td>
<td>G</td>
<td>39.4</td>
<td>-3 (+4.7)</td>
</tr>
<tr>
<td>PCPO</td>
<td>G</td>
<td>45.1</td>
<td>-6.2 (+0.7)</td>
<td>PCPO</td>
<td>G</td>
<td>39.9</td>
<td>+0.5 (+4.9)</td>
</tr>
</tbody>
</table>

a Tm values of duplexes (6.0 µM) measured in 50 mM sodium phosphate buffer, pH 7, with 0.1 M NaCl, heating rate of 1 °C/min, errors are ± 1 °C. b ΔTm = Tm (modified duplex) – Tm (unmodified duplex). ΔTm values in brackets = Tm (PhO,G, PhPhO,G, TCPO,G, PCPO,G modified duplex) – Tm (PhO,G modified duplex).

The most significant difference in Tm values occurred when the O-linked adducts were placed opposite an abasic site (THF) and the truncated 10-mer sequence (~2). Both PhPhO,G and NaphO,G showed a dramatic stabilizing effect opposite THF (ΔTm ~ +4.5 °C compared to G, ~ +7.5 °C compared to PhO,G). These results contrasted with our previous findings for PhO,G, which was destabilizing opposite THF (ΔTm = -3.4 °C). Opposite N = -2, the PhPhO,G lesion stabilized the 2-base bulge (ΔTm = +6.6 °C compared to X = G, +11.0 °C compared to X = PhO,G). However, this stabilizing influence was not observed for NaphO,G compared to X = G. For the chlorinated analogs a small stabilizing effect was observed for PCPO,G opposite THF that was not observed with TCPO,G (ΔTm =
+0.7 °C and −3.0 °C respectively). This trend is repeated opposite \( N = −2 \), and again, a slight stabilization effect was observed for \( \text{PCPOG} \) only \((ΔT_m = +0.5 \ °C \ and \ −1.2 \ °C \) respectively). Importantly, the stabilization effect observed for \( \text{PCPOG} \) is within the experimental error for the \( T_m \) measurement, and cannot be considered significant when comparing the \( T_m \) values to \( X = \text{G} \). However, upon comparison to \( \text{PhOG} \), chlorination seemed to induce some duplex stabilization, as \( \text{PCPOG} \) demonstrated higher \( T_m \) values when \( N = \text{THF} \) and \( N = −2 \) \((ΔT_m = +4.1 \ °C \ and \ +4.9 \ °C \) compared to \( X = \text{PhOG} \)). The stabilizing effect was also more pronounced with \( \text{PCPOG} \), as \( \text{TCPG} \) did not show any change in \( T_m \) compared to \( N = \text{PhOG} \), in both cases (Table 2-1).

2.3.3. CD Spectral Measurement Results.

CD spectral overlays (Figure 2-10) of the modified \( \text{NarI} \) duplexes illustrate comparisons between \( \text{PhOG} \) (solid black trace) to the duplexes containing \( \text{PhPhOG} \) (dotted blue traces), \( \text{NaphOG} \) (dashed red traces), \( \text{TCPG} \) (dotted light green traces), and \( \text{PCPG} \) (dashed dark green traces). In general, the duplexes all gave rise to typical B-form CD patterns, with a major positive band at ~ 275 nm, a negative band at ~ 240 nm, and crossover at ~ 260 nm.\(^{116,117} \) Specific changes in CD spectra were compared to \( \text{PhOG} \), based on rigorous structural characterization of this adduct in previous work.\(^{77} \) Within the B-form duplex structures, the \( \text{PhOG} \) lesion adopts a B conformation opposite C with the adduct present in an \textit{anti}-conformation.\(^{77} \) Watson-Crick (W-C) H-bonding with C is maintained, and the phenoxy ring is positioned in the major groove exposed to solvent.\(^{77} \) In contrast, \( \text{PhOG} \) is present in a \textit{syn} orientation opposite G and takes on a wedge (W) conformer with the phenoxy ring residing in the minor groove.\(^{77} \) Comparisons between the five modified duplexes were carried out by examining the major positive band centered at ~ 275 nm. Changes in wavelength and intensity for this band are given in Table 2-2.
Figure 2-10: CD spectral overlays of modified Narl duplexes with $X = O$-linked C8-dG adduct opposite: (a) $N = C$, (b) $N = G$, (c) $N =$ THF, (d) $N = -2$; $X = \text{PhO}_G$ (solid black lines), $X = \text{PhPhO}_G$ (dotted blue lines), $X = \text{NaphO}_G$ (dashed red lines), $X = \text{TCPO}_G$ (dotted light green lines) and $X = \text{PCPO}_G$ (dashed dark green lines).

In general, the $\text{PhPhO}_G$ lesion displayed a profound increase in amplitude for all four duplex structures. The relative intensity ($I_{rel}$) values increased by roughly two-fold for $N = C$, G and THF and 1.5-fold for $N = -2$, compared to $X = \text{PhO}_G$. Hyperchromicity of the positive CD band is indicative of an increase in π-stacking interactions. $^{45,118}$ In addition, significant blue shifts (5-8 nm) were observed in the wavelength maximum ($\lambda_{\text{max}}$) when compared to the Narl duplexes containing $\text{PhO}_G$. A blue shift in this positive band has been attributed to DNA bending or kinking. $^{119,120}$ In contrast, the Narl duplexes containing $\text{NaphO}_G$ gave rise to $I_{rel}$ and $\lambda_{\text{max}}$ values that were more comparable to the duplex containing $\text{PhO}_G$ (Table 2-2, Figure 2-10). $I_{rel}$ values of 1.6 and 1.5 were observed
for NaphO$_2$G paired opposite THF and C, suggesting an increase in π-stacking interactions for these duplex structures. The duplex with NaphO$_2$G paired opposite C also exhibited a large blue shift (6 nm) indicating DNA bending, as noted for the duplexes containing PhPhO$_2$G. The CD spectrum of NaphO$_2$G paired opposite G was almost identical to the corresponding spectrum for PhO$_2$G (Figure 2-10B), suggesting that NaphO$_2$G also adopts the W conformation within the G mismatch. CD traces obtained for both TCPO$_2$G and PCPO$_2$G were quite similar to PhO$_2$G (Figure 2-10). Although PCPO$_2$G showed hyperchromicity opposite N = G ($I_{rel} = 1.5$), all other traces showed minimal hyperchromic effects that were within experimental error (Table 2-2).

**Table 2 - 2: CD Peak Intensities and Shifts of C8-Phenoxy-G Modified NarI Duplexes**

<table>
<thead>
<tr>
<th>X</th>
<th>N</th>
<th>$\lambda_{max}$ (nm)$^a$</th>
<th>$I_{[mdeg]}$ b</th>
<th>$\Delta \lambda_{max}$ (nm)$^c$</th>
<th>$I_{rel}$ [mdeg]$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhPhO$_2$G</td>
<td>C</td>
<td>279</td>
<td>0.68</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>271</td>
<td>0.85</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>THF</td>
<td>272</td>
<td>0.86</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>−2</td>
<td>275</td>
<td>0.98</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaphO$_2$G</td>
<td>C</td>
<td>273</td>
<td>0.99</td>
<td>−6</td>
<td>1.5</td>
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<tr>
<td></td>
<td>G</td>
<td>270</td>
<td>1.01</td>
<td>−1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>THF</td>
<td>269</td>
<td>1.33</td>
<td>−3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>−2</td>
<td>272</td>
<td>1.08</td>
<td>−3</td>
<td>1.1</td>
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<tr>
<td>TCPO$_2$G</td>
<td>C</td>
<td>278</td>
<td>0.78</td>
<td>−1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>271</td>
<td>0.86</td>
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</tr>
<tr>
<td></td>
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<td>0.98</td>
<td>−2</td>
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<tr>
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<td>−2</td>
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<td>0.99</td>
<td>−2</td>
<td>1.0</td>
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<tr>
<td>PCPO$_2$G</td>
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<td>0.78</td>
<td>−3</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>271</td>
<td>1.04</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>THF</td>
<td>271</td>
<td>0.86</td>
<td>−1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>−2</td>
<td>272</td>
<td>1.04</td>
<td>−3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$ Wavelength at maximum intensity; $^b$ Intensity at $\lambda_{max}$; $^c$ Difference in the wavelength of positive band between $X = $ PhPhO$_2$G, NaphO$_2$G, TCPO$_2$G or PCPO$_2$G and the control duplex with $X = $ PhO$_2$G; $^d$ Relative intensity between the positive band for $X = $ PhPhO$_2$G, NaphO$_2$G, TCPO$_2$G or PCPO$_2$G versus the control duplex with $X = $ PhO$_2$G.
The chlorinated analogs also showed minimal or non-existant blue shifts in their absorbance maxima (Table 2-2). While TCPO\textsuperscript{G} exhibited minimal changes in absorbance maxima ($\Delta\lambda_{\text{max}}$ between 0 nm and 2 nm), PCPO\textsuperscript{G} did show a small amount of blue shift in absorbance for N = C and N = −2 ($\Delta\lambda_{\text{max}} = -3$ nm). However, in comparison to PhO\textsuperscript{G}, the effect was negligible suggesting that duplexes for the chlorinated analogs are in a similar state of distortion to duplexes formed by PhO\textsuperscript{G}.

2.3.4. Discussion of Structural Studies on O-linked Adducts

Our previous studies on the single-ringed PhO\textsuperscript{G} adduct demonstrated that the free PhO\textsuperscript{dG} nucleoside adopts a syn conformation which reduces steric strain between the phenolic ring and deoxyribose sugar moiety.\textsuperscript{88} In the lowest energy syn structure (determined by DFT calculations at the B3LYP/6-311+G(2df,p) level), the biaryl ether component is planar.\textsuperscript{88} Despite the syn-planar preference of PhO\textsuperscript{dG}, when inserted into the NarI sequence at G\textsubscript{3}, the O-linked PhO\textsuperscript{G} lesion adopts an anti conformation to produce the major groove B-type duplex structure when paired opposite C.\textsuperscript{77} Furthermore, the adduct becomes skewed with respect to G.\textsuperscript{88} In the present study, O-linked C8-dG adducts derived from 4-hydroxybiphenyl (PhPhO\textsuperscript{dG}), 2-naphthol (NaphO\textsuperscript{dG}), trichlorophenol (TCPO\textsuperscript{dG}), and pentachlorophenol (PCPO\textsuperscript{dG}) were similarly incorporated into G\textsubscript{3} of NarI to determine how attachment of a second phenyl ring, as well as chlorination to PhO\textsuperscript{G} impacted duplex structure. The additional phenyl ring increases adduct lipophilicity and extends conjugation of the aromatic system, which enhance potential π-stacking interactions within the duplex. Chlorination also increases the lipophilicity of the phenyl ring, but adds bulky groups that affect steric interactions between the adduct and neighboring nucleobases, while providing no additional stacking support. The syn-planar NaphO\textsuperscript{dG} was expected to engage in greater π-stacking interactions than PhPhO\textsuperscript{dG} because biphenyl systems are generally twisted to relieve
steric interactions.\textsuperscript{121} In addition, MD calculations predict the phenyl ring in PhO\textsubscript{G} to be skewed compared to guanine in the duplex (Figure 2-8).\textsuperscript{88} As planarity was expected to play an important role in establishing stacking interactions, the skew of the biphenyl moiety was expected to disfavor incorporation into the helix.\textsuperscript{122}

The use of thermal melting temperatures ($T_m$), to compare stabilities of the adducted NarI duplexes to unmodified controls, displayed significant changes when the O-linked C8-dG adducts were paired opposite the abasic site (N = THF, Table 2-1). Purine bases are known to remain within the duplex π-stack when paired opposite an abasic site,\textsuperscript{123} and the pairing of modified bases with THF allows assessment of π-stacking interactions in the absence of H-bonding.\textsuperscript{124} Both PhPhO\textsubscript{G} and NaphO\textsubscript{G} displayed significant duplex stabilization when paired opposite THF ($\Delta T_m = +8.2, +7.8$ °C vs. PhO\textsubscript{G}, Table 2-1), suggesting that the adducts adopted a syn structure to favor π-stacking interactions within the duplex. The pronounced stabilizing effect was not observed with TCPO\textsubscript{G} opposite THF ($\Delta T_m = -3.0$ °C); however, PCPO\textsubscript{G} showed slight stabilization ($\Delta T_m = +4.1$ °C vs. PhO\textsubscript{G}, Table 2-1). The CD spectral data (Figure 2-10C, Table 2-2) were also in support of favorable π-stacking interactions for PhPhO\textsubscript{G} and NaphO\textsubscript{G} opposite THF, as relatively large hyperchromic effects ($I_{rel} = 2.1$ and 1.6) were observed for both of these duplex structures compared to the duplex containing PhO\textsubscript{G}. Hyperchromicity was not observed for TCPO\textsubscript{G} and PCPO\textsubscript{G} ($I_{rel} = 1.1, 1.0$), which suggests that stacking interactions were similar to PhO\textsubscript{G}.

The $T_m$ and CD data for PhPhO\textsubscript{G} and NaphO\textsubscript{G} paired opposite THF also suggested the propensity of these lesions to form a stacked S conformer when paired opposite C which is similar to the behavior of structurally similar N-linked C8-dG adducts produced by arylamine carcinogens.\textsuperscript{44,45} As addressed in section 2.1, N-linked adducts can exhibit conformational heterogeneity to produce all three conformers (B, S, and W) upon hybridization to the normal pyrimidine partner C.\textsuperscript{73} In the S conformer, the N-linked C8-
dG adduct adopts a syn conformation rupturing W-C H-bonding with C. The lipophilic aryl moiety is incorporated into the π-stack of the duplex which shields it from the aqueous bulk solvent. In this conformation the opposing C is flipped out of its natural intrahelical position into a solvent exposed extrahelical environment. Because this conformation lacks H-bonding interactions and is stabilized by π-stacking interactions between the C8-aryl moiety and the duplex, we propose that a duplex pairing the modified base with THF is a reasonable model for the S conformer, and adducts that can stabilize this duplex will show syn preference.

By comparing duplex $T_m$ values with the lesion paired opposite N = C vs. THF (Table 2-1), the potential for the S conformer to contribute to conformational heterogeneity opposite C can be established. For example, both unmodified G and PhO-G will not produce the S conformer because comparison of the $T_m$ values provides a difference of 17.9 °C and 10.5 °C respectively (Table 2-1). Clearly W-C H-bonding interactions with the opposing C play significant roles in duplex stability for unmodified G and PhO-G. In sharp contrast, comparison of the $T_m$ values for PhPhO-G and NaphO-G provides a difference of only ~2 °C.

The chlorinated analogs also show interesting effects when analyzing duplex stability opposite THF. TCPO-G produces a difference of −3.3 °C while PCPO-G shows a difference of −0.2 °C between N = C and N = THF. It appears that hydrogen bonding does not influence the stability of PCPO-G, as observed for PhPhO-G. TCPO-G is destabilized slightly, although the effect is not as pronounced as PhO-G or G. Since duplex stability remained constant in the absence of H-bonding, we hypothesize that hydrophobic effects from the chlorinated phenyl ring forces some syn preference, flipping the chlorinated ring inside the helix when N = THF. Furthermore, the lack of an opposing base may facilitate this process by decreasing steric effects upon entry of the chlorinated ring to the helical environment. Sterics were postulated to be a barrier to entry for the
chlorinated analogs and this may explain why the difference in duplex stability between 
\( N = \text{C} \) and \( N = \text{THF} \) for \( X = {\text{POPO}} \) is comparable to \( X = {\text{NaphO}} \) and \( X = {\text{PhPhO}} \) (Table 2-1). Although \( {\text{POPO}} \) may adopt the \textit{syn} orientation to minimize destabilizing solvent interactions, there remains a lack of supplementary \( \pi \)-stacking interactions. The stabilizing influence observed was correspondingly less when \( N = \text{THF} \), compared to \( {\text{PhPhO}} \) and \( {\text{NaphO}} \) adducts.

The CD spectral data is also supportive of contributions from the \( S \) conformer when \( N = \text{THF} \) and indicates increased \( \pi \)-stacking and significant helical bending for both duplex structures pairing \( {\text{PhPhO}} \) and \( {\text{NaphO}} \) with \( \text{C} \) compared to the corresponding \( {\text{PhO}} \) duplex (\( I_{\text{rel}} = 1.9 \) and 1.5, \( \Delta \lambda_{\text{max}} = -5, -3 \text{ nm} \), Table 2-2, Figure 2-10). The chlorinated CD spectra show small blue shifts for \( {\text{TCPO}} \) and \( {\text{PCPO}} \) (\( \Delta \lambda_{\text{max}} = -2 \text{ nm} \)) but do not show any hyperchromicity compared to \( {\text{PhO}} \) which is consistent with the lack of additional \( \pi \)-stacking interactions compared to \( {\text{PhO}} \).

The \( T_m \) data exhibited significant differences for the adducted \( \text{NarI} \) strands when paired opposite the truncated 10-mer oligonucleotide (\( N = -2 \), Table 2-1). Certain \( N \)-linked \( \text{C8-dG} \) adducts stabilize a 2-base bulge in duplex structures. This has correlated with their ability to form stable \( S \) conformers and induce \( -2 \) frameshift mutations.\textsuperscript{72,84} For the \( O \)-linked \( \text{C8-dG} \) adducts, stabilization of the truncated duplex was only observed for the \( {\text{PhPhO}} \) lesion (\( \Delta T_m = +6.6 \text{ °C vs. G}; +11.0 \text{ °C vs. PhO} \), Table 2-1). In contrast, \( {\text{NaphO}} \) was unable to stabilize the 2-base bulge when compared to the unmodified duplex (Table 2-1). This result was surprising but suggests that the fused-planar napthol ring within the bulge cannot extend deep enough into the \( \pi \)-stack of the duplex to stabilize the structure. \( {\text{PCPO}} \) and \( {\text{TCPO}} \) displayed no significant differences compared to unmodified \( \text{G} \), did not stabilize the bulge structure and are, therefore, not predicted to act as frame-shift mutagens (Table 2-1).
The CD spectral data (Figure 2-10D, Table 2-2) also showed hyperchromicity and significant bending, indicative of π-stacking and an S conformation, for PhPhO\textsubscript{G} (N = −2, I\textsubscript{rel} = 1.4, Δλ\textsubscript{max} = −6 nm) but not for the corresponding duplex with NaphO\textsubscript{G}, TCPO\textsubscript{G}, or PCPO\textsubscript{G} (I\textsubscript{rel} = 1.1, 1.0, 1.1) when compared to PhO\textsubscript{G}. Both sets of data suggest that PhPhO\textsubscript{G} is the only adduct that could be predicted to act as a frame-shift mutagen.

The T\textsubscript{m} data for PhPhO\textsubscript{G} and NaphO\textsubscript{G} paired with G exhibited no significant differences (Table 2-1). However, the CD data was informative and provided almost identical spectra for the mismatched duplexes containing PhO\textsubscript{G} and NaphO\textsubscript{G} (Figure 2-10B). Previous studies supported a W conformer for PhO\textsubscript{G} paired with G which positions the phenoxy ring in the minor groove of the helix. This conformation favors Hoogsteen H-bonding by the syn-adduct with anti-G in the opposite strand, as indicated by MD simulations. Solid-state evidence of a natural G mismatch also favors Hoogsteen H-bonding between the two G bases. Based on the CD spectral overlays depicted in Figure 2-10B, it is reasonable to conclude that NaphO\textsubscript{G} also adopts the W conformer opposite G. In contrast, the CD spectral data for PhPhO\textsubscript{G} paired with G (Figure 2-10B) resembled the corresponding CD data for PhPhO\textsubscript{G} paired with the abasic site (THF, Table 2-2). Both duplexes exhibit significant hyperchromicity (I\textsubscript{rel} = 2.1, Table 2-2) pointing to π-stacking interactions between the biphenyl moiety and the helix.

PCPO\textsubscript{G} and TCPO\textsubscript{G} both exhibit significant destabilizing effects opposite N = G (ΔT\textsubscript{m} = −8.7 °C and −8.5 °C, Table 2-1) although CD data was also very similar to PhO\textsubscript{G}. For chlorinated adducts in the W conformation, the lipophilic moiety would remain partially solvent exposed. This energetically unfavorable interaction may explain the destabilization observed opposite G (ΔT\textsubscript{m} = −6.5 °C compared to PhO\textsubscript{G}, Table 2-1), but the data is insufficient to substantiate an argument for adoption of the W conformation by TCPO\textsubscript{G} or PCPO\textsubscript{G}.
2.3.5. Fluorescence Studies with $^{\text{CNBPO}}G$.

Although $T_m$ and CD data for the $^{\text{PhPhO}}G$ adduct gave evidence for the adoption of the S conformer in DNA, it was desirable to use a fluorescent $^{\text{PhPhO}}G$ analog to probe the local environment of the adduct in different duplexes. To this end, the 4-cyanophenylphenoxy-dG adduct ($^{\text{CNBPO}}dG$) was synthesized. Synthesis of the corresponding phosphoramidite and incorporation into NarI proceeded without difficulty and fluorescent data was collected on the individual nucleoside and the modified DNA strands.

2.3.5.1 $^{\text{CNBPO}}dG$ Nucleoside Fluorescent studies

Studies on the behavior of $^{\text{CNBPO}}dG$ in aqueous buffer solutions were conducted to determine absorbance and emission maxima of the individual nucleoside in various solvents but also to investigate the effect of rigidity and temperature on fluorescent behavior. These values were then used to compare the effects observed in the various NarI duplexes (N = G, C, THF, −2) in an effort to establish the local environment of $^{\text{CNBPO}}G$ in these sequences. Interestingly, because the ether linkage between guanine and the cyanobiphenyl moiety does not conjugate these two molecules, fluorescent signal changes were only attributed to structural variations on the biphenyl component. This is in contrast to previously studied C-linked adducts that contained conjugation through the entire molecule.\textsuperscript{104}
Figure 2 - 11: $\lambda_{\text{ex}}$ (max) and $\lambda_{\text{em}}$ (max) for $\text{CNBPO}dG$ in water (black traces), DMSO (dashed red traces), ACN (dotted brown traces) and THF (dashed blue traces).

In the first set of experiments, fluorescence of $\text{CNBPO}dG$ was compared in aqueous buffer, DMSO, ACN, and THF (Figure 2-11). Although there was minimal change in $\lambda_{\text{ex}}$ max, large shifts were observed in $\lambda_{\text{em}}$ max. These spectral shifts proved to be very useful to probe the local adduct environment as the excited state demonstrated solvent sensitivity. Similar D-A biphenyl systems possess ground-state structures with an angular distribution between the rings close to planarity ($0^\circ$).

**Table 2 - 3: Solvatochromic spectral properties of $\text{CNBPO}dG$**

<table>
<thead>
<tr>
<th>Solvent (ε)</th>
<th>$\lambda_{\text{ex}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Delta\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (78.5)</td>
<td>308</td>
<td>408</td>
<td>7958</td>
</tr>
<tr>
<td>DMSO (48.9)</td>
<td>311</td>
<td>391</td>
<td>6579</td>
</tr>
<tr>
<td>ACN (37.5)</td>
<td>308</td>
<td>386</td>
<td>6560</td>
</tr>
<tr>
<td>THF (7.39)</td>
<td>312</td>
<td>373</td>
<td>5242</td>
</tr>
</tbody>
</table>

$^a$ Dielectric constants at 25 °C, taken from Reference $^{127}$. $^b$Stokes shift ($\Delta\nu$) is calculated as $(1/\lambda_{\text{ex}} - 1/\lambda_{\text{em}})$.

Table 2-3 illustrates fluorescent excitation maxima, emission maxima, and Stokes shifts for $\text{CNBPO}dG$ in different solvents. As solvent polarity decreases, $\lambda_{\text{ex}}$ remains relatively similar but $\lambda_{\text{em}}$ blue-shifts significantly. Furthermore, the Stokes shift decreases in less polar solvents. We suspect that in polar environments, $\text{CNBPO}dG$ has an
approximately planar geometry which is supported by previous work on biphenyl molecules, and MD simulations on PhO\textsuperscript{dG} demonstrating planar geometry with respect to the phenyl ring and guanine base. In non-polar solvents, a large blue shift in $\lambda_{em}$ max (38 nm) consistent with a higher energy excited state was observed. Non-polar solvents such as THF lack the ability to stabilize the charge separated excited state from the donor acceptor pair (Figure 2-12), and the adoption of the planar excited state becomes more energetically unfavorable.

![Diagram of ground state and charge separated excited state](image)

**Figure 2 - 12: CT fluorescent relaxation state for CNBPO\textsuperscript{dG} which is stabilized by polar solvents.**

Figure 2-11 demonstrates the solvent dependant emission changes of CNBPO\textsuperscript{dG}. As solvent polarity decreases, the $\lambda_{em}$ max changes from 408 nm in aqueous buffer to 372 nm in THF (Figure 2-11) which has been observed previously for D-A biphenyl systems. Lack of solvent H-bonding does not appear to affect the ground state of the nucleoside as $\lambda_{em}$ max remained constant (Table 2-3). However, non-polar solvents cannot stabilize the CT process and decreased solvent stabilization of the excited state is exemplified by the lower Stokes shifts in non-polar solvents, a common trend for highly polarized excited states (Table 2-3). Lack of stabilization to the CT excited state requires more energy to attain planarity, and a higher energy, hypsochromic shift in emission peak is the result (Figure 2-12).
Upon the addition of glycerol to the solution, $\lambda_{em}$ decreased in intensity and blue shifted (Figure 2-13). Increasing the percentage glycerol in the solution continued this trend, and 80% glycerol solution gave the largest blue shift in fluorescence ($\Delta \lambda_{max} = -12$ nm) with a quenched signal ($I_{rel} = 0.48$, Table 2-3, Figure 2-13). Heating the solution from 20 °C to 70 °C increased the emission intensity by 3 fold (Table 2-5). Similar trends have been observed previously for fluorescent C-linked adducts studied in the Manderville laboratory.\(^{104}\)

**Table 2 - 4: Spectral changes of $\textit{CNBPO}dG$ with glycerol addition**

<table>
<thead>
<tr>
<th>% Glycerol</th>
<th>$\lambda_{max}$</th>
<th>Intensity</th>
<th>$\Delta \lambda_{max}$</th>
<th>$I_{rel}$</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>412</td>
<td>234</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>406</td>
<td>212</td>
<td>-6</td>
<td>0.90</td>
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<tr>
<td>60</td>
<td>403</td>
<td>150</td>
<td>-9</td>
<td>0.64</td>
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<tr>
<td>80</td>
<td>399</td>
<td>113</td>
<td>-12</td>
<td>0.48</td>
</tr>
</tbody>
</table>

**Figure 2 - 13: Fluorescence emission changes with glycerol addition. Initial scan is solid black trace, increasing glycerol concentrations are denoted with dotted traces.**

Upon increasing viscosity, excited states are known to have geometries similar to that of the ground state, as solvent rigidity increases the barrier to rotation.\(^{129}\) The slightly twisted geometry proposed for the ground state of $\textit{CNBPO}dG$ in water needs to rotate to obtain a planar CT excited state. Increasing viscosity inhibits the adoption of this highly-planar quinoid-type geometry upon excitation.\(^{130,131}\) The data suggests that the excited state of $\textit{CNBPO}dG$ remains twisted in glycerol, which requires more energy to obtain planarity, ultimately resulting in the blue shift observed in the spectrum (Figure 2-
Increasing the temperature of the glycerol solution decreases the barrier to rotation and the CT planar excited state is generated more easily by $^{\text{CNBPO}}dG$. The fluorescence once again red-shifts as conjugation increases the intensity of emission (Figure 2-14).

### Table 2 - 5: Fluorescent emission intensity changes with increasing temperature

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Intensity</th>
<th>$I_{\text{rel}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>195</td>
<td>1.71</td>
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<tr>
<td>40</td>
<td>215</td>
<td>1.88</td>
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<tr>
<td>50</td>
<td>247</td>
<td>2.16</td>
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<tr>
<td>60</td>
<td>300</td>
<td>2.63</td>
</tr>
<tr>
<td>70</td>
<td>380</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Figure 2 - 14: Temperature effects on $^{\text{CNBPO}}dG$ fluorescence emission. Initial scan is solid black trace and temperature increases are denoted with dashed and dotted traces.

2.3.5.2. Fluorescent Experiments on $^{\text{CNBPO}}G$ at G3 of NarI.

To probe the local environment of $^{\text{CNBPO}}G$ in DNA, the adduct was incorporated into NarI at G3 using the same method as other O-linked adducts. $T_m$ experiments were performed, and the $^{\text{CNBPO}}G$ produced thermodynamic influences on duplex stability that were similar to the $^{\text{PhPhO}}G$ adduct (Table 2-5).

Although there is virtually no difference between the two adducts opposite N = C and G ($\Delta T_m = -0.5, -1$ °C, Table 2-5), small increases were observed for the duplexes containing N = A (+1.7 °C), T (+2.9 °C), THF (+1.4 °C) and −2 (1.7 °C). These small stabilizing influences were attributed to increased conjugation provided by the cyano group on the biphenyl system. As the π-electron system is extended, this effect increases stacking capability thus stabilizing duplexes that have a more intimate
association with the biphenyl moiety. However, this effect is minimal which suggests that orientations of the \( \text{CNBPO} \) are similar to \( \text{PhPhO} \) and supports the use of \( \text{CNBPO} \) as a fluorescent probe for the conformation of \( \text{PhPhO} \) in DNA.

Table 2 - 6: Thermal Parameters of \( \text{CNBPO} \) and \( \text{PhPhO} \) Modified \text{Narl} Oligonucleotides

<table>
<thead>
<tr>
<th>( X )</th>
<th>( N )</th>
<th>( T_m ) (°C)(^a)</th>
<th>( \Delta T_m )(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CNBPO} )</td>
<td>C</td>
<td>52.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>C</td>
<td>52</td>
<td>-0.5</td>
</tr>
<tr>
<td>( \text{PhPhO} )</td>
<td>G</td>
<td>53.2</td>
<td>-1</td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>G</td>
<td>52.2</td>
<td>-1</td>
</tr>
<tr>
<td>( \text{PhPhO} )</td>
<td>A</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>A</td>
<td>50.7</td>
<td>1.7</td>
</tr>
<tr>
<td>( \text{PhPhO} )</td>
<td>T</td>
<td>50.4</td>
<td></td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>T</td>
<td>53.3</td>
<td>2.9</td>
</tr>
<tr>
<td>( \text{PhPhO} )</td>
<td>THF</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>THF</td>
<td>51.9</td>
<td>1.4</td>
</tr>
<tr>
<td>( \text{PhPhO} )</td>
<td>-2</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>( \text{CNBPO} )</td>
<td>-2</td>
<td>47.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a\)\( T_m \) values of duplexes (6.0 \( \mu \text{M} \)) measured in 50 mM sodium phosphate buffer, pH 7, with 0.1 M NaCl, heating rate of 1 °C/min, errors are ± 1 °C. \(^b\)\( \Delta T_m = T_m (\text{CNBPO}) - T_m (\text{PhPhO}) \)

Fluorescent data for the \( \text{CNBPO} \) adduct opposite \( N = (C, G, \text{THF and } -2) \) is shown in Figure 2-15. Interestingly, once \( \text{CNBPO} \) is incorporated into a duplex, a considerable blue shift occurs for both absorbance and fluorescence compared to the nucleoside in aqueous buffer (Table 2-3). Fluorescent data for the modified single strand was quite similar to nucleoside results. \( \text{CNBPO} \) incorporated into a duplex shifted \( \lambda_{ex} \) from 308 nm to 297 nm and \( \lambda_{em} \) from 399 nm to 383 nm (Figure 2-15, Table 2-7). Furthermore, significant emission quenching was observed of almost 9 fold upon DNA incorporation. Substantial evidence of fluorescent quenching from DNA strands exists in the literature as close associations between fluorescent nucleobase and neighboring bases influences optical properties.\(^4\) For example, 2-aminopurine exhibits substantial
quenching when inserted into DNA, which may explain the general quenching observed upon insertion of $^{\text{CNBPO}}$G into DNA.\textsuperscript{134}

![Figure 2 - 15: Fluorescent traces for NarI modified duplexes when $X = ^{\text{CNBPO}}$G. $N =$ none (single strand, solid black trace), $N = C$ (light blue trace), $N = G$ (dark blue trace), $N = \text{THF}$ (dark red trace), $N = -2$ (green trace).](image)

Substantial hypsochromic shifts of $\lambda_{\text{ex}}$ (5-11 nm, Table 2-7) upon duplex formation, were observed for $N = C$, $G$, THF, and $-2$ compared to $N =$ none. Furthermore, $\lambda_{\text{ex}}$ of the modified single strand matched $\lambda_{\text{ex}}$ max for the nucleoside in water ($\lambda_{\text{ex}} = 308$ nm) which suggested the biphenyl adduct moiety was present in an aqueous environment and contained a similar twist angle to the nucleoside. Upon duplex formation, the blue-shifted $\lambda_{\text{ex}}$ suggested that $^{\text{CNBPO}}$G adopted a more twisted ground state conformation. Steric interactions within the confines of the duplex were
hypothesized to promote this effect, forcing the twist angle between phenyl rings to become increasingly similar to unsubstituted biphenyls. These molecules typically have a twist angle between phenyl rings of 30° to 40° to relieve steric tension.\textsuperscript{135} Fluorescence data on sterically-forced, twisted D-A biphenyl systems show similar blue shifts in excitation spectra compared to planar derivatives, and the degree of rotation influences the hypsochromicity produced.\textsuperscript{128}

Table 2 - 7: Changes in spectroscopic properties for modified Narl X = \textsuperscript{CNBPG} opposite N = (C, G, THF, -2 and none)

<table>
<thead>
<tr>
<th>X \textsuperscript{CNBPG}</th>
<th>N</th>
<th>(\lambda_{\text{ex}}) (nm)</th>
<th>(\Delta\lambda_{\text{max,ex}}) (nm)</th>
<th>(\lambda_{\text{em}}) (nm)</th>
<th>(\Delta\lambda_{\text{max}}) (em)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>297</td>
<td>-11</td>
<td>387</td>
<td>-9</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>297</td>
<td>-11</td>
<td>388</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>303</td>
<td>-5</td>
<td>388</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>THF</td>
<td>299</td>
<td>-9</td>
<td>383</td>
<td>-13</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>308</td>
<td>0</td>
<td>396</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

When comparing \textsuperscript{CNBPG} paired with different opposing strands (Table 2-7), the largest blue shifts were observed when \(N = C\) and \(N = G\) (\(\Delta\lambda_{\text{ex}}\) -11 nm) which suggested that these duplexes produced the greatest amount of biphenyl twist. Interestingly, the smallest blue shift was observed when \(N = -2\) (Table 2-7). This effect was attributed to lower steric interactions within the bulge promoting a more planar orientation between the phenyl rings.
Thermal melting ($T_m$) data strongly suggested that $^{PhPhOG}$ adopts an S conformation opposite $N = THF$ which inserts the biphenyl moiety inside the helix. Ground-state fluorescent data suggests that the phenyl rings become more twisted in relation to each other, and the degree of twist is much greater compared to the single strand ($N = none$, Table 2-6). In general, emission spectra in duplexes also underwent blue-shifting, as $\lambda_{em}$ (max) resembled values obtained for $^{CNBPO}dG$ in non-polar solvents (Table 2-5). The emission spectra were not suggestive of a B conformation for any of the duplexes analyzed, as placement of the biphenyl moiety in the major groove upon B conformation would produce similar emission data to single strand ($N = none$).

The fluorescent data was very surprising because the biphenyl adduct was assumed to require adoption of a planar geometry to maximize $\pi$-stacking within the helix and stabilize the S conformation, as proposed for the corresponding N-linked adduct, ABP. Our results suggest that planarity between phenyl rings is not necessary for helix insertion of $^{PhPhOG}$.

Results from MD simulations indicate that the single-ringed $^{PhOG}$ adduct is skewed compared to G in the B and W conformations (Figure 2-16). It is plausible that the secondary phenyl ring in $^{PhPhOG}$ adopts a skewed conformation in relation to the primary phenyl ring attached to guanine, which is supported by fluorescent data, but
maintains planarity with G and the neighboring bases facilitating π-stacking and stabilizing the duplex in the S conformation ($T_m$ data, Table 2-1). This may also explain increased syn conformation for $\text{PhPhO}_G$ compared to $\text{NaphO}_G$. If the rings remain twisted inside the helix and do not require planarity for π-stacking, insertion of $\text{PhPhO}_G$ into the helix becomes increasingly energetically favorable. The position of the second phenyl ring deeper into the helix and π-stacking interactions from the second phenyl ring stabilize the S conformer and give $\text{PhPhO}_G$ more syn character.

2.3.6. Relevance of Structural and Fluorescent Experiments.

To provide further rationale for the biological significance of our findings regarding phenoxy ring expansion of O-linked C8-dG adducts, it is informative to draw comparison to the structurally similar N-linked C8-dG adducts derived from arylamine carcinogens. Direct comparison has been made between the mutagenicity of 2-napthalamine (NA), 4-aminobiphenyl (ABP), 2-aminofluorene (AF), and 2-acetylaminofluorene (AAF) using the Ames Salmonella test. At a concentration of ~25 µg/plate the number of revertants using human liver microsomes for bioactivation follows the order AAF > AF >> ABP >> NA. Both AAF and AF produced > 5000 revertants while ABP generated ~ 650 and NA ~ 150 revertants. All amines undergo bioactivation to produce N-linked C8-dG adducts, whose relative mutational frequencies depend strongly on neighboring bases surrounding the lesion site which can dictate adduct conformation. In general, the ability of N-linked C8-dG adducts to adopt the S conformer is regarded as pro-mutagenic. It is well established that the N-linked C8-AAF lesion strongly favors the syn conformation in duplex DNA to produce S and W structures while the corresponding C8-AF adduct can exhibit conformational heterogeneity, adopting the B conformer in addition to S and W.
The Cho laboratory has also examined the conformational preference of the N-linked C8-ABP adduct and has observed a strong dependence on flanking sequence. Earlier NMR evidence, with C8-ABP adduct in a 15-mer flanked by 5′-T and 3′-A favored a B conformer (90-95% of the total duplex structure) with the biphenyl positioned in the major groove of the duplex. A minor conformer (5-10%) was also detected which appeared consistent with the S conformer. More recent 19F NMR evidence with the C8-ABP adduct flanked by C bases indicated ~ 60% S while 40% remained in the B conformation. In the same sequence, the C8-AF adduct produced 90% S and only 10% B. The methylene linker in AF increases biphenyl planarity which increases the mutagenicity of AF compared to ABP and provides a stronger preference for the C8-AF adduct to produce the pro-mutagenic S conformer. To the best of our knowledge, the N-linked C8-NA adduct has not been incorporated site-specifically into an oligonucleotide substrate for conformational analysis. However, our findings indicate that the O-linked PhPhOG lesion has greater π-stacking ability than NaphOG in the NarI duplex, and fluorescent analysis indicates that the biphenyl system does not require planarity between rings for helix insertion. These results suggest that sufficient ring extension into the DNA helix and planarity of the second ring with neighboring nucleobases are responsible for stabilizing a syn orientation of O-linked polycyclic adducts. These effects may explain why ABP shows ~ 5-fold increase in mutagenicity compared to NA in the Ames assay.

Upon analysis of chlorinated O-linked analogs, that are known to form from PCP, the $T_m$ and CD data suggest that TCPOG and PCPOG can adopt a syn conformation and stack when N = THF although the effect is not as pronounced compared to the polycyclic adducts. Any syn preference for PCPOG can only be attributed to solvent effects as π-stacking was similar to PhOG, shown by the CD data. Although PCPOG and TCPOG contain bulky moieties on the phenyl ring, the inherent hydrophobicity appears to overcome
energy requirements to flip the adduct into a syn conformation inserting the polychlorinated phenyl moiety into the helix. Although the data is suggestive of this effect, it is difficult to confirm this conformation based on the $T_m$ and CD data alone. Furthermore, the chlorinated phenyl ring is predicted by MD simulations to be skewed with respect to G. Obtaining planarity to allow stacking would require an energetic cost for this process, not examined for this Thesis. It is possible that the syn preference observed for $^{\text{PCPO}}\text{G}$ may produce some S conformation in duplexes. Based on fluorescent data with $^{\text{CNBPO}}\text{G}$, planarity of the ring immediately adjacent to guanine does not appear to be necessary for helix insertion which would aid incorporation of bulky chlorinated adducts on solvent effects alone.

Although S conformers are considered more mutagenic, they are also more easily recognized and repaired. Current study in the Manderville laboratory is analyzing interactions of various $C$ and $O$-linked adducts with polymerase enzymes to observe if adduct interference with enzyme-DNA interactions can provide supplemental mutagenic mechanisms that expand on structural results. It is possible to predict potential mutagenic effects from $^{\text{NaphO}}\text{G}$ and $^{\text{PhoO}}\text{G}$ as $T_m$, CD and fluorescence data demonstrated significant changes. Although chlorinated adducts provide some compelling evidence for adoption of a syn conformation and helix insertion, the lack of definitive results for structural orientation of $^{\text{TcPO}}\text{G}$ and $^{\text{PCPO}}\text{G}$ in DNA requires further experimentation to elucidate more definitive conformational evidence.

2.4. Chlorinated Adduct Mass Spectrometry Experiments.

To further probe the mechanistic carcinogenicity of polychlorinated $O$-linked adducts, $^{\text{PhO}}\text{G} \ (8)$, $^{\text{4ClPhO}}\text{dG} \ (9)$, $^{\text{2ClPhO}}\text{dG} \ (10)$, and $^{\text{DClPhO}}\text{dG} \ (11)$ were synthesized in addition to the previously analyzed nucleosides according to established procedures. The family of adducts used in MS studies are shown in Figure 2-17. The first
experiments performed on these adducts studied the influence of Cl-substitution on the hydrolytic stability of the O-linked adducts in aqueous acidic media and in the gas-phase using positive ionization ESI-MS.\textsuperscript{88} Depurination to form an abasic site occurs frequently with many C-linked guanine adducts and has shown some correlations to their carcinogenicity.\textsuperscript{139,140} We were curious about influences of chlorination on hydrolytic stability for O-linked adducts. Interestingly, in aqueous media, the unsubstituted PhO\textsubscript{dG} adduct was the most susceptible to hydrolysis. Increased Cl-substitution diminished the rate of hydrolysis by lowering N7 basicity.\textsuperscript{88} However, in the gas-phase, where ESI-MS experiments target isolate monoprotonated nucleosides and determine their relative stability to deglycosylation, PhO\textsubscript{dG} was the least reactive.\textsuperscript{88} This result was attributed to electronic factors which altered the site of protonation in chlorinated adducts from N7 to N3 and inhibited deglycosylation. The effects of chlorination on nitrogen atom pK\textsubscript{a} suggested that formation of an abasic site is not involved in toxic mechanisms for polychlorinated O-linked adducts.

During the course of these experiments, we also analyzed the nucleoside adducts using negative ionization ESI-MS and further explored CID of the modified nucleobases in MS\textsuperscript{3}. These experiments uncovered a unique ESI-MS CID pathway for O-linked C8-dG adducts. To provide mechanistic insight into the CID results, the corresponding C8-benzyloxy-dG adduct (\textsuperscript{BnO}dG 7, Figure 2-22b) was synthesized, and density function theory (DFT) calculations were performed.

Furthermore, as PCP is known to form O-linked C8-dG adducts \textit{in vitro}, which may be implicated in the toxicity of this compound, it was desirable to incorporate both PCPO\textsubscript{G} and BnO\textsubscript{G} into the 12-mer NarI oligonucleotide (Figure 2-17c) to determine CID behavior within DNA. The goal of these experiments was to investigate influences of Cl-substitution on gas-phase reactivity for O-linked C8-dG adducts and to determine ESI-MS CID patterns which could provide diagnostic SRM pathways for effective dose
biomarkers following exposure to polychlorinated POPs. The nucleosides and modified oligonucleotides were subjected to sequential tandem mass spectrometry (MSn) experiments, and their pathways were fully characterized. These results were very informative as differences in fragmentation were observed between the nucleosides and the oligonucleotides.

Figure 2 - 17: a) DNA adduction pathways for chlorophenols and structures of O-linked C8-dG adducts used in MS study. b) Structure of BnO<sub>d</sub>G (6). c) Nar1 12-mer oligonucleotide sequence and position (G<sub>3</sub>) of PCPO<sub>G</sub> and BnO<sub>G</sub> incorporation.

2.4.1 MS<sup>2</sup> Fragmentation Pathways for Modified Nucleosides.

CID of the O-linked C8-dG adducts by ESI<sup>-</sup>-MS produced daughter ions indicating sugar loss (-116, deglycosylation) and phenyl radical loss to produce a distonic ion with m/z 281. Figure 2-18 shows the ESI<sup>-</sup>-MS<sup>2</sup> spectrum of TCPO<sub>d</sub>G. The nucleoside parent ion at [M – H]<sup>-</sup> = 460 is shown, and daughter ions from CID at m/z 344 (-116), 308 (-152) and 281 (-179) were observed.
Figure 2 - 18: ESI-MS² spectrum of a) $^{TCP}\text{dG}$ and b) $^{BnO}\text{dG}$.

The fragment peak at $m/z$ 308 represents loss of the sugar moiety followed by loss of HCl ($116 + 36 = 152$). The fragment at $m/z$ 281 represents loss of the TCP ring system ($C_6H_2Cl_3 = 179$) suggesting homolytic fission of the ether linkage to release the neutral TCP radical. This observation prompted synthesis of $^{BnO}\text{dG}$ (7, Figure 2-17b) as this nucleoside adduct was expected to undergo homolytic fission of the ether linkage, given the stability of the resonance-stabilized benzyl radical. Figure 2-18b shows the ESI-MS² spectrum of $^{BnO}\text{dG}$. As anticipated, the adduct ($[M - H]^-$ = 372) showed preferential loss of 91 (benzyl radical) to afford the distonic ion at $m/z$ 281.

Figure 2 - 19: Relative abundance of sugar loss versus phenyl radical loss for O-linked C8-dG adducts in the ESI-MS² spectra.
For the family of chlorinated O-linked C8-dG adducts (5-11), Figure 2-19 shows the relative abundance of sugar loss versus phenyl radical loss in the ESI−-MS² spectra. For the unsubstituted adduct Ph-O-dG (8), sugar loss was the predominate fragmentation pathway (~22%) with radical loss contributing ~2%. The chlorinated derivatives were more susceptible to CID. A substantial increase in sugar loss was observed for 4ClPhO-dG (9, ~82%) that was also accompanied by an increase in radical loss (~10%) compared to PhO-dG. Further addition of chlorine atoms, resulted in a decrease of sugar loss and an increase in radical loss (Figure 2-19). The ortho-derivative 2ClPhO-dG (10) displayed an almost 3-fold increase in percentage of radical loss (~28%) compared to 4ClPhO-dG (9) suggesting a positional effect of chlorination on radical fragmentation. The fully substituted adduct PCPO-dG (6) provided ~57% phenyl radical loss and 25% sugar loss (Figure 2-19a). The abundance of radical loss from PCPO-dG was the most prominent and was comparable to the extent of benzyl radical loss from BnO-dG (~60%, not shown).

2.4.2. MS³ Fragmentation Pathways for Modified Nucleosides

The ESI−-MS² experiments yielded fragments for the nucleobase anions through sugar loss and the distonic ion at m/z 281 from phenyl radical loss. Protonated adducts produced [M − 116 + H]⁺ ions preferentially. These product ions were isolated inside the ion trap producing sequential tandem MS³ mass spectra to further characterize diagnostic fragmentation pathways for the family of O-linked C8-dG adducts. The ESI−-MS³ spectrum of the distonic ion with m/z 281 is shown in Figure 2-20. Product ions were observed at m/z 192, 165 and 122. The m/z 192 ion is the major product observed during CID of 8-OH-dG using negative ionization. This product results from cleavage of the C-C and C-O bonds in the deoxyribose sugar ring and is defined as an S1 type fragment. The ion at m/z 165 represents complete sugar loss to produce the distonic
ion of the nucleobase. This species then showed loss of 43 to produce \( m/z \) 122 which was attributed to loss of isocyanic acid (HNCO) a characteristic loss from the six-membered ring of deprotonated guanine.\(^{141}\) Product ions representing sugar loss (165), and sugar fragmentation (192), were also accompanied by ions at \( m/z \) 166 and 191, respectively. This observation is indicative of hydrogen transfer processes occurring during these fragmentation pathways.

![Figure 2 - 20: ESI−MS\(^3\) spectrum of \( m/z \) 281.](image)

The ESI-MS\(^3\) spectra for both the deprotonated and protonated nucleobase of \( \text{BnOG}, \ \text{TCPOG}, \ \text{and PCPOG} \) are shown in Figure 2-21. The ESI−-MS\(^3\) spectrum of \( \text{BnOG} \) shows loss of the benzyl radical to afford \( m/z \) 165 as the major fragment (Figure 2-21a). Other fragments include \( m/z \) 239 and 213 which are attributed to losses of ammonia (-17) and isocyanic acid (-43) respectively, from the nucleoside. Interestingly, the protonated \( \text{BnOG} \) species does not show benzyl radical loss (Figure 2-21b). Instead, product ions corresponding to the benzylic cation (\( m/z \) 91) and a second major fragment at \( m/z \) 180 corresponding to the neutral loss of benzene were observed. Similar
fragmentation pathways have been reported previously for other monoprotonated benzyl ethers.\textsuperscript{142}

Figure 2 - 21: MS\textsuperscript{3} spectra of negatively (a,c,e) and positively (b,d,f) charged nucleobases of $^{\text{BHO}}\text{G}$ (a and b), $^{\text{TCPo}}\text{G}$ (c and d) and $^{\text{PCpo}}\text{G}$ (e and f).
The ESI$^-$-MS$^3$ spectra of TCPO$^G$ (Figure 2-21c) and PCPO$^G$ (Figure 2-21e) showed losses of 36 (HCl) a known CID fragment of polychlorinated phenols. For TCPO$^G$, the [M – HCl – H]$^-$ peak at m/z 308 demonstrated loss of 43 (isocyanic acid) from the guanine component to afford the ion at m/z 265 (Figure 2-21c). For PCPO$^G$, the [M – HCl – H]$^-$ peak at m/z 376 showed loss of HCl (−36) to afford m/z 340 and isocyanic acid to afford m/z 333. The [M – 2HCl – H]$^-$ peak at m/z 340 then showed loss of isocyanic acid to afford the peak at m/z 297. Surprisingly, both negatively-charged Cl-substituted O-linked C8-G adducts did not show the distonic ion at m/z 165 corresponding to phenyl radical loss. This observation was in sharp contrast to the ESI$^+$-MS$^3$ spectra of both modified bases (Figures 2-21d and 2-20f) where the ion at m/z 167 representing phenyl radical loss from the protonated nucleoside was prominent. Other fragment peaks resulted from losses of ammonia (-17) and NHCNH (-42) which are characteristic losses from the 6-membered ring of guanine in the positive mode.$^{144}$

2.4.3. MS$^n$ of PCPO$^G$-Nar$^I$ Nucleotides.

The ESI$^-$-MS spectrum of PCPO$^G$-Nar$^I$ is shown in Figure 2-22a. CID of PCPO$^G$-Nar$^I$ was achieved by isolating the m/z 767.6 peak ([M – 5H]$^5$). The resulting ESI$^-$-MS$^2$ spectrum is shown in Figure 2-22b. Interestingly, phenyl radical loss from PCPO$^G$-Nar$^I$ was not observed. Instead, CID led to loss of PCPO$^G^-$ to produce the ion at m/z 412 containing the characteristic Cl-isotope pattern of the PCP moiety (Figure 2-22b). Fragmentation of the oligonucleotide produced fragment ions of m/z 500, 641, and 750 which were consistent with strand cleavage at the abasic site created by loss of PCPO$^G^-$ (Figure B-6, Appendix B).$^{94}$ CID of the ion at m/z 412 (Figure 2-22c) showed losses of 36 (HCl) and 43 (isocyanic acid), consistent with the ESI$^-$-MS$^3$ spectrum of the negatively charged nucleobase (Figure 2-21e). This behavior was in contrast to the fragmentation pathways observed for BrO$^G$-Nar$^I$ (B7-B9, Appendix B). Similar to BrO$^d$G, the major
fragmentation pathway involved benzyl radical loss (-91) to produce the distonic ion at \( G_3 \) of Narl with \([M - 7H]\)^7\(^-\) = 525.1. The ESI-MS\(^2\) spectrum of \(^{\text{BnO}}\text{G-Narl}\) also showed the distonic ion nucleobase at \( m/z \) 165 and the deglycosylated base at \( m/z \) 256 (Figure B8).

Figure 2-22: a) ESI-MS spectrum of \(^{\text{PCPO}}\text{G-Narl}\). b) ESI-MS\(^2\) spectrum of the \([M - 7H]\)^7\(^-\) ion of \(^{\text{PCPO}}\text{G-Narl}\). c) MS\(^3\) spectrum of \( m/z \) 412 (\(^{\text{PCPO}}\text{G}\)).
2.4.4. Discussion of CID Results for Nucleosides and Nucleotides.

The fragmentation pathways observed for O-linked C8-dG adducts are summarized in Scheme 2-2. ESI–MS$^2$ experiments in the negative mode produced ions resulting from two major fragmentation pathways, deglycosylation to release the negatively charged nucleobase and phenyl radical loss to afford the distonic ion with $m/z$ 281 (Scheme 2-2a). A mechanistic proposal for sugar loss involves nucleophilic attack by the oxyanion of the 5′-OH group on C1′ to release the negatively charged nucleobase and generate the neutral sugar containing a 5′-O-C1′ bond.\textsuperscript{144}

\textbf{Scheme 2 - 2: ESI-MS CID Pathways for O-linked C8-dG adducts by a) negative mode, b) positive mode and c) PCPO-G-NarI by negative mode}
Using positive ionization, ESI⁺-MS² experiments showed preferential sugar loss to produce the protonated nucleobase and the neutral sugar moiety (Scheme 2-2b). This effect is due to the positive charge on the base which makes it a better leaving group, and encourages nucleophilic attack on the electron deficient anomeric carbon. Significant differences in the CID patterns of the charged nucleobases were then observed for ESI-MS³ experiments using the two polarities (Figure 2-21). In the negative mode, both TCPOG⁻ and PCPOG⁻ failed to undergo phenyl radical loss to generate the distonic ion with m/z 165. Instead, losses of HCl (-36) and isocyanic acid (-43) were observed. In the positive mode, radical loss (RL) to afford the distonic ion with m/z 167 was a prominent product ion (Figures 2-21d and 2-21f). It appears that distonic radical formation is influenced by the polarity of the charge and the proximity of the charge site to the radical site. During MS³ experiments, the adducts are deglycosylated. The positive charge resides on the guanine nucleobase component, and is in close proximity to the site of radical cleavage at the ether linkage. Distonic ion formation was not observed for the negatively charged nucleobase, suggesting that the stability of the distonic radical anion with the charge at the α-position is less than the nucleoside distonic radical anion containing the negative charge localized on the sugar.

Upon fragmentation of the PCPOG-NarI oligonucleotide, deglycosylation was the predominate pathway and evidence for PCP radical loss was not detected in the MS² spectrum (Figure 2-22b). This result was surprising as fragmentation was expected to follow the pattern observed for the free nucleoside PCPOdG in which phenyl radical loss was the preferred pathway (Figure 2-22a). However, in DNA, loss of a nucleobase is proposed to proceed through nucleophilic attack on C1' by the negatively charged 3'-phosphate group (Scheme 2-2c). The favorable position of the nucleophilic 3'-phosphate group for attack at C1', coupled with the leaving-group ability of the electron-deficient PCPOG, precludes competitive phenyl radical loss. This finding was contrasted
by ESI\(^{-}\)-MS\(^2\) experiments with \(\text{BnO-G-NarI}\) which showed preferential benzyl radical loss (Appendix B, Figure B-8). Identification of this pathway for \(\text{BnO-G-NarI}\) confirmed that benzyl radical loss competed effectively with abasic site formation in DNA, but loss of chlorinated phenyl radicals did not compete effectively with loss of \(\text{PCPO-G}^{-}\) within \(\text{PCPO-G-NarI}\).

### 2.4.5. Rationale for Phenyl Radical Loss

To explain the effect of chlorine substitution on distonic radical ion production for the O-linked C8-dG nucleosides, DFT calculations on C-H bond cleavage energies were performed. Table 1 shows \(\Delta G\) and \(\Delta\Delta G\) energy values for formation of the phenyl radical by hydrogen abstraction according to the isodesmic reaction depicted in Scheme 2-3. Upon chlorination of the benzene ring, \(\Delta G\) values increased from 146.2 kJ/mol for the unsubstituted benzene to a maximum value of 162.0 kJ/mol for 1,3,5-trichlorobenzene, suggesting that increased chlorination disfavored phenyl radical formation (Table 2-5).

**Scheme 2 – 3: Isodesmic reaction used to calculate the reaction free energies of hydrogen abstraction from chloro-substituted benzenes at the B3LYP/6-31G(d) level**

![Scheme 2](image)

Substituent effects on phenyl radical reactivity have been studied in detail by the Kenttämaa laboratory using MS experiments.\(^{146,147}\) These studies demonstrated that halogen substituents (F and Cl) increase the electron affinity (EA) of the phenyl radical, making it more electrophilic and more reactive toward H-atom abstraction processes.
Cioslowski and colleagues utilized DFT calculations to determine the impact of Cl-substitution on phenyl radical formation from polychlorinated benzenes. The Cl-substituents were predicted to make C–H cleavage less favorable by as much as 3.8 kcal/mol. The destabilizing influence of the Cl-substituents was ascribed to inductive electronic effects. Chlorines in ortho and para positions were destabilizing while meta chlorines exerted a slightly stabilizing impact on aryl radical formation. Thus, in our calculations, trichlorobenzene exerts the greatest destabilizing influence on phenyl radical formation (ΔG = 162.0 kJ/mol, Table 2-5) as it contains two chlorines in ortho positions and one para position. Formation of pentachlorophenyl radical is more favorable (ΔG = 157.4 kJ/mol, Table 2-5) as the starting benzene has two m-chlorines that stabilize the resulting phenyl radical. Combined, these results all strongly suggest that homolytic bond cleavage of the O-linked C8-dG nucleoside adducts was not driven by stability of the aryl radical product.

**Table 2 – 8: Calculation of free energy at the B3LYP/6-31G(d) level for hydrogen abstraction from benzene and chlorinated benzenes**

<table>
<thead>
<tr>
<th>Benzene</th>
<th>ΔG (kJ/mol)</th>
<th>ΔΔG (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsubstituted</td>
<td>146.2</td>
<td>0</td>
</tr>
<tr>
<td>monochloro</td>
<td>150</td>
<td>3.7</td>
</tr>
<tr>
<td>1,3-dichloro</td>
<td>156</td>
<td>9.8</td>
</tr>
<tr>
<td>1,3,5-trichloro</td>
<td>162</td>
<td>15.8</td>
</tr>
<tr>
<td>pentachloro</td>
<td>157.4</td>
<td>11.2</td>
</tr>
</tbody>
</table>

*ΔΔG was calculated by (ΔG<sub>chlorinated benzenes</sub> – ΔG<sub>unsubstituted benzene</sub>)*

Further calculations were conducted to analyze the bond dissociation energy (BDE) of homolytic bond cleavage for the O-linked nucleosides according to Scheme 2-4.
Scheme 2 - 4: Homolytic cleavage reaction between the bond connecting the C8-oxygen atom and the (chlorosubstituted or unsubstituted) phenyl moiety of the adducts used to calculate BDE at the B3LYP/6-31G(d) level.

Increased chlorination decreased the BDE significantly (263.1 kJ/mol for Ph-O-dG (8) vs 244.1 kJ/mol for PCP-O-dG (6), Table 2-6. The decrease in BDE with increased chlorination correlated with the abundance of phenyl radical formation from the gas phase ESI-MS data (Figure 2-19).

Table 2 - 9: Bond-dissociation energies (BDEs) associated with homolytic cleavage of the bond connecting the C8–oxygen atom and the (chlorosubstituted or unsubstituted) phenyl moiety of the O-linked C8-dG adducts using B3LYP/6-31G(d) calculations.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>BDE (kJ/mol)</th>
<th>ΔBDE (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph-O-dG (8)</td>
<td>263.1</td>
<td>19</td>
</tr>
<tr>
<td>4-Cl-Ph-O-dG (9)</td>
<td>264.5</td>
<td>20.4</td>
</tr>
<tr>
<td>2,4-DCP-O-dG (11)</td>
<td>261.6</td>
<td>17.5</td>
</tr>
<tr>
<td>TCP-O-dG (5)</td>
<td>251.6</td>
<td>7.5</td>
</tr>
<tr>
<td>PCP-O-dG (6)</td>
<td>244.1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Cioslowski and colleagues also utilized DFT calculations to determine the impact of Cl-substitution on phenyl radical formation from polychlorinated benzenes following C-Cl homolytic bond cleavage. In this instance, steric interactions between adjacent...*
chlorine atoms encouraged the C-Cl cleavage reaction by up to 6.6 kcal/mol.\textsuperscript{148} Thus, for the polychlorinated O-linked C8-dG nucleoside adducts in the current study, we propose that steric strain between \textit{ortho} chlorines and the C8-ether oxygen atom facilitates homolytic bond cleavage, driving phenyl radical loss through relief of steric strain.

\textbf{2.4.6. Implications for O-linked C8-dG Adduct Detection.}

DNA adduction by chlorophenols may proceed by two pathways involving P450-mediated metabolism to generate quinone electrophiles,\textsuperscript{51} or by peroxidase activation to afford electrophilic phenoxyl radicals (Figure 2-17a).\textsuperscript{35} Increased Cl-substitution of the phenol ring increases the lifetime of the phenoxyl radical\textsuperscript{53} and increases phenol toxicity.\textsuperscript{145} Structure-activity relationships show that phenol toxicity correlates with phenoxyl radical production.\textsuperscript{96} The peroxidase pathway is very effective at facilitating DNA adduction by PCP. 32P-Postlabeling experiments carried out \textit{in vitro} show that treatment of calf thymus DNA with PCP in the presence of horseradish peroxidase (HRP)/H\textsubscript{2}O\textsubscript{2} affords \~3600 adducts/10\textsuperscript{7} nucleotides.\textsuperscript{149} This treatment generates the O-linked C8-dG adduct \textit{PCPO}\textsubscript{dG} as the major species.\textsuperscript{56} Human exposure to chlorophenols may occur through direct contact or by indirect exposure through \textit{in vivo} hydroxylation of PCBs\textsuperscript{23} which are established carcinogens.\textsuperscript{150} The PCBs are present as complex mixtures, and it is predicted that highly-substituted analogs will undergo peroxidase-mediated metabolism to afford persistent phenoxyl radical species that can attach to the C8-site of G within DNA as noted for PCP. However, at present, \textit{in vivo} evidence for O-linked C8-dG adduct formation by chlorophenols is lacking.

The results of the MS study outline the ESI-MS fragmentation patterns for chlorinated O-linked C8-dG adducts. In the negative mode, the nucleoside adducts undergo CID to produce the distonic ion with \textit{m/z} 281. This ion increases in abundance upon increased chlorination due to steric destabilization of the nucleoside. The distonic
ion appears unique to O-linked adducts as unmodified bases and most other modified purines show preferential sugar loss (−116). The diagnostic m/z 281 fragment allows use of selective reaction monitoring (SRM) experiments to detect O-linked C8-dG adducts produced by polychlorinated phenols in complex mixtures. Furthermore, complete characterization of fragmentation pathways for O-linked modified nucleosides and modified DNA, demonstrated important differences in gas phase reactivity. The fragmentation pathways elucidated allow O-linked adduct detection by SRM regardless of procedural differences in enzymatic digestion methods that can produce both nucleosides and nucleotides. Nucleosides are used for effective dose biomarkers for exposure to other carcinogens, and we believe that the information gained from our fragmentation studies will aid the detection of O-linked adducts from future in vivo studies.

2.5. Conclusions.

T
m
, CD, fluorescence, MD and mass spectrometry studies on O-linked adducts provided informative findings for conformational preferences of these adducts in DNA. This class of adducts has not been extensively studied, and the work reported in Chapter 2 probed the behavior of O-linked adducts in DNA upon formation. Previous studies on the simplest O-linked adduct PhOG provided initial studies, from which expansion of the ring system could continue the structural investigation on more biologically relevant adducts. Our results strongly suggested that PhPhOG can adopt an S conformation in the helix based on the propensity to stabilize THF and bulge duplexes. Although this effect was more pronounced than NaphOG, a small stabilizing effect was still observed with NaphOG opposite THF. These experiments strongly suggested that proper placement of the second ring deep into the helix was the major stabilizing influence for
PhPhOG to π-stack and adopt an S preference. Promotion of the S orientation for PhPhOG may provide a rational for mutagenic outcomes observed for N-linked analogs of aromatic amine carcinogens ABP and NA. At first, this was surprising, because planarity between phenyl rings was thought to be necessary for PhPhOG to stack in the helix, making NapOOG more energetically favored to adopt the S conformer. Fluorescence studies demonstrated that the phenyl rings remained twisted in relation to one another upon stacking in the helix. MD simulations also showed some initial skew of the first phenyl ring, compared to G, suggesting that the second ring could be twisted in relation to the first but remain planar compared to guanine, permitting proper π-stacking. Since PhPhOG does not require planarity to stack, its increased S preference becomes much more plausible energetically.

The most biologically relevant adducts, the chlorinated O-linked moieties, also showed some propensity to stack within the helix an effect that could only be attributed to increased lipophilicity compared to PhOG. Although bulk and steric effects were present upon introduction of chlorine atoms, the high degree of lipophilicity appeared to favor some syn preference. A lack of extra stacking interactions did not provide increased stability, necessary for S conformation adoption, to the extent observed for PhPhOG and NapOOG, but results for N = THF were suggestive of some syn conformational preference. Although the structural data provided is insufficient to predict mutagenic outcomes, further studies currently underway in the Manderville laboratory should confirm structural preferences, and enzyme interactions for all of the adducts studied herein. Ultimately, these results provide a possible mechanism for the mutagenicity observed from polychlorinated phenols in the environment.

MS studies on the polychlorinated adducts demonstrated an interesting homolytic cleavage pathway producing a consistent radical fragment at \( m/z = 281 \) for the entire
family of O-linked adducts. This pathway is unique, to the best of our knowledge, for O-linked DNA adducts, and would be useful for biomarker detection and identification of these species following polychlorinated phenol exposure. Furthermore, dissociation pathways were elucidated in DNA providing unambiguous identification for O-linked adduct formation regardless of sample preparation and digestion strategy (nucleosides or nucleotides). The fragmentation pathways provided may facilitate future detection of O-linked adducts from in vivo exposures which would increase the biological relevance of this family of covalent lesions.

2.6. References


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Chapter 3.

Hydroxyl Radical-Induced Oxidation of a Phenolic C-Linked C8 Guanine Nucleoside Produces Reactive Secondary Products that may Contribute to Phenol Toxicity
Note on Reproduction of Work

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3.1. Introduction

In addition to the $O$-linked adducts formed by phenols through the production of phenolic radicals studied in Chapter 2, the ambident reactivity of these radicals also allows formation of $C$-linked adducts at the C8 site of dG.\textsuperscript{1} $C$-linked phenol adducts are more well studied in the literature and have been previously analyzed in the Manderville laboratory for their spectroscopic and oxidative properties.\textsuperscript{1} This chapter focuses on expanding the oxidative chemistry of $C$-linked phenolic adducts and investigates the toxicological significance of secondary oxidative products, providing further mechanistic insight into phenol mediated toxicity.

An important aspect in the toxicological pathways of most phenols involves oxidation. Oxidative processes in cells are responsible for many beneficial and detrimental effects.\textsuperscript{2,3,4} They are necessary for energy generation and enzyme function\textsuperscript{5} but can cause cytotoxicity if not properly controlled.\textsuperscript{6} Oxidation products vary in their composition and consist of radical and non-radical species, which are implicated in several toxic mechanisms.\textsuperscript{7,8} Metal ions carry out oxidative process directly,\textsuperscript{7} and oxidation is a key reaction pathway for CYP 450 enzymes, which can lead to bioactivation.\textsuperscript{8} Peroxidase enzymes are known to catalyze the one-electron (e$^-$) oxidation of phenols producing phenoxy radicals that can generate toxic effects by several different pathways.\textsuperscript{9} One mechanism involves the generation of reactive oxygen species (ROS) and is described as futile thiol pumping.\textsuperscript{10} In this mechanism, the enzymatically generated phenoxy radicals are reduced by thiols to generate thyl radicals that stimulate the generation of new ROS, such as $O_2$$^•$ and hydroxyl radical (HO$^•$) (Figure 3-1).\textsuperscript{10}
Figure 3 - 1: Phenol oxidation by peroxidase enzymes generates phenoxy radicals and initiates futile thiol pumping which generates secondary ROS.

ROS generated from phenoxy radicals are implicated in many different adverse cellular effects but can interact directly with genetic information, potentiating genotoxic pathways that have been well characterized and involve multiple targets. Hydrogen abstraction by ROS can occur at several sites within DNA, both from the nitrogenous bases and also from the sugar phosphate backbone.

Figure 3 - 2: Hydrogen abstraction from the 4' site of deoxyribose in a DNA strand by hydroxyl radical leads to strand cleavage. One consequence of oxidative stress on DNA

Abstraction of a hydrogen atom from ribose or deoxyribose produces a carbon-based sugar radical that rearranges to cause strand scission (Figure 3-2). This process occurs at most of the available hydrogens on deoxyribose, but non-selective radicals show preference for abstraction from the 5' and 4' positions. Preferential abstraction
occurs due to solvent accessibility and close proximity of 5’ and 4’ hydrogens to the electron-withdrawing oxygen atom.\textsuperscript{12}

Hydroxyl radicals also abstract hydrogens from nucleobases to cause oxidative damage directly to the coding molecules. Most often, oxidation occurs at deoxyguanosine (dG) to afford 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxo-dG) which is commonly used as a biomarker for oxidative damage.\textsuperscript{13,14} Guanine is most often the target of oxidative attack due to its electron richness and low oxidation potential in comparison to the other bases (Chapter 1, Table 1-1).\textsuperscript{15}

\textbf{Figure 3 - 3:} Oxidation of 8-oxo-dG (dOG) to produces spiroiminohydantoin (Sp) and guanidinohydantoin (Gh) lesions following covalent reaction with water. Covalent reaction with phenolate anion generates the tricyclic 4,5-PhO-dOG adduct.\textsuperscript{26}

Furthermore, 8-oxo-dG has been shown to cause mutagenesis and has been implicated in aging.\textsuperscript{16} \textit{In vitro} studies have demonstrated the ability of 8-oxo-dG to cause
G-T tranversion mutations, but in vivo studies show a relatively low percentage of mutations (< 10%). This is due to repair enzymes in the cell which remove 8-oxo-dG lesions efficiently by base excision repair (BER).\(^\text{17}\) An interesting feature of 8-oxo-dG is its decreased oxidation potential (\(E^o = 0.74\) V vs NHE\(^\text{18}\)) relative to dG (\(E^o = 1.29\) V vs NHE\(^\text{15}\)) making certain phenoxyl radicals (\(E^o \sim 1\) V vs NHE) capable of facilitating dOG oxidation.\(^\text{19}\) This produces spiroiminohydantoin (Sp) and guanidinohydantoin (Gh) lesions that were introduced in Chapter 1 of this Thesis and are depicted again in Figure 3-3.\(^\text{20}\) These lesions have demonstrated increased mutagenicity compared to 8-oxo-dG.\(^\text{21}\) Poor recognition of these hyperoxidized products by existing enzyme systems may account for this observation; the literature reports that Sp and Gh are very poorly repaired in vivo.\(^\text{20}\)

DNA melting studies indicate that duplexes containing Sp or Gh paired opposite A, T, C, or G show destabilization relative to duplexes containing a G:C pair.\(^\text{22}\) Additionally, computational study of the Sp nucleoside revealed that the two rings of the Sp and Gh structures are essentially planar and perpendicular to each other while displaying severely restricted conformational freedom.\(^\text{23,24}\) MD simulations predicted Sp to sit in the major groove of B-form DNA, although both syn and anti orientations have been reported for Sp.\(^\text{24}\) Both Sp and Gh act as strong blocks to replication by Klenow fragment, although the small amount of full length strand synthesized showed insertion of G and A exclusively opposite Sp which would also lead to mutations.\(^\text{25,22}\) These structural results highlight the increased mutagenicity of secondary oxidation products and validates the study of these secondary reactions. The lesions produced from secondary oxidative processes are less commonly encountered, more difficult to repair, distort DNA, block polymerase enzyme function, and are, therefore, more mutagenic.\(^\text{26,27}\)
The Burrows laboratory has shown that phenolates can also react covalently with oxidized 8-oxo-dG to generate the tricyclic 4,5-PhO-dOG product shown in Figure 3-3. As many DNA binding proteins such as topoisomerases and repair enzymes contain tyrosine in their active sites, covalent attachment by phenolate to form DNA-protein cross-links (DPCs) was investigated as an additional mechanism of toxicity by oxidative stress from phenol exposure. These reactions depended on attack from a nucleophilic phenolate anion, and the reaction was facilitated by the low oxidation potential of dOG. Inhibition of protein function through covalent attachment was concluded to have potential implications in the pathogenesis of oxidative stress. Highlighted throughout these studies was the combination of different phenolic toxicity pathways to produce DPC lesions.

In addition to perpetuating oxidative stress in cells, phenoxyl radicals produced by oxidation of parent phenols can also react directly with DNA by addition to the C8-site of dG. Carbon C-linked adducts are produced and have been observed from in vivo studies (Chapter 1, Scheme 1-1). The C-linked adducts shown in Figure 3-4 are also generated by mutagenic diazoquinones and are structurally related to a family of 8-aryl(Ar)-dG adducts produced by aryl hydrazines, polycyclic aromatic hydrocarbons (PAHs), estrogens, and nitroaromatics. These adducts share a common intermediate the carbon centered aryl radical. C-linked phenol adducts at the C8 site of dG are created by one-electron (e-) reactions of phenols with ROS or enzymatic catalysis to form the carbon radical intermediate which reacts with the C8 site of guanine preferentially. Adduct formation, in this way, is thought to potentiate mutagenicity and carcinogenicity observed in these compounds.
Figure 3 - 4: Some examples of C-linked adducts at the C8 position of deoxyguanosine produced by various suspected carcinogens. A) polyaromatic hydrocarbons B) estradiol C) nitroaromatics D) fungal toxin ochratoxin A.

Phenols undergo another important metabolic oxidative pathway that may contribute to their toxic effects. Peroxidase enzymes, bacterial enzymes, and CYP P450 catalyze hydroxylation of phenols to afford catechols. This reaction pathway is important for eliminating polychlorinated pesticides in the environment, lipophilic PAHs from the body, and metabolising endogenous compounds such as estrogens and dopamine neurotransmitters. Despite the benefits of catechol creation, they easily undergo redox cycling to generate semiquinone radicals and quinone electrophiles, in conjunction with the generation of superoxide radical anions (O$_2^-$, Figure 3-5). Redox reactions are somewhat beneficial and provide a wide variety of necessary biological and chemical functions. These functions include the electron transport (ETC), photosynthesis and posttranslational modification of proteins. Redox reactions are also
essential in the detoxification of pro-oxidant species and ROS by anti-oxidants like vitamins and flavanoids.\textsuperscript{44,45}

Figure 3-5: Redox cycling mechanism of catechols initiated by cellular oxidants to produce ROS that damage cellular molecules and the formation of semi-quinones and quinones that form covalent adducts with DNA and proteins.

Quinones produced from redox reactions with catechols are highly reactive compounds that generate ROS themselves through redox cycling which further increases oxidative stress in the cell and causes damage to cellular macromolecules (Figure 3-5).\textsuperscript{46,47} In addition to oxidative mechanisms, partially substituted quinones can function as arylating agents. Cellular nucleophiles, including thiols on cysteine residues in proteins, glutathione (GSH),\textsuperscript{48} and detoxifying agents such as \textit{N-acetylcysteine} (NAC), form covalent quinone–thiol Michael adducts.\textsuperscript{49} Covalent attachment by quinones depletes glutathione stores and hinders proper protein function.\textsuperscript{50} Protein inhibition by quinone adduct formation has been used beneficially in antibiotic therapies. The drug Geldanamycin binds selectively to a chaperone protein (Hsp 90) and inhibits its
Many cellular anti-oxidants like glutathione contain sulphur nucleophiles, and depletion of anti-oxidant molecules by covalent attachment perpetuates oxidative stress.

Protein binding also affects other cellular processes including DNA replication and DNA repair which can result in genotoxicity. The drug Nevirapine causes severe idiosyncratic reactions that result from its metabolism, formation of a reactive quinone methide, and inhibition of a CYP 450 enzyme through adduct formation. The altered metabolism of other compounds from this CYP 450 inhibition has been shown to lead to cytotoxicity.

![Metabolism of dopamine by CYP 450 to form quinone molecule and covalent adduct formation with A and G.](image)

**Figure 3 - 6: Metabolism of dopamine by CYP 450 to form quinone molecule and covalent adduct formation with A and G.**

In addition to the indirect genotoxic effects described above, electrophilic quinones can react at nucleophilic sites in DNA causing direct genotoxicity. Formation of quinone-DNA adducts has been implicated in the etiology of Parkinson's disease from the formation of a dopamine-guanine adduct upon oxidation (Figure 3-6). Other quinone metabolites from benzene and estrogen have been shown to form direct DNA adducts, and it is strongly suspected that these quinones are implicated in the development of adverse health effects following exposure to elevated levels of these compounds. Catechol production has been implicated in estrogen-mediated
genotoxicity and carcinogenesis\textsuperscript{57} as well as cardiotoxicity from anthracycline chemotherapeutics.\textsuperscript{58} 

The Manderville laboratory has been studying the properties of phenolic C-linked nucleoside adducts because of their expected role in phenol-mediated toxicity.\textsuperscript{59-61} Electrochemical measurements of the C-linked para-isomer [8-(4''-hydroxyphenyl)-dG (p-PhOH-dG)] showed that attachment of the phenolic moiety to the C8 site of dG considerably lowers the oxidation potential of the base, making it susceptible to further oxidation.\textsuperscript{60} 

![Figure 3 - 7: Oxidation of p-PhOH-dG to form C-linked polymeric products through the intermediacy of the phenoxy radical.\textsuperscript{57}](image)

These studies, performed by Weishar and colleagues, focused on the expansion of redox properties for C8 aryl adducts in the generation of biomaterials. It was observed that all aryl adducts studied lowered the oxidation potential compared to dG.\textsuperscript{59} Interestingly, when p-PhOH-dG was subjected to electo-oxidation, phenolic radical coupling products resulted.\textsuperscript{62} The other adducts in the study all produced well established Sp and Gh lesions, typical of oxidation of dG in the presence of water.\textsuperscript{62}
Thus, similarly to dOG, \(^{63}\) \(p\)-PhOH-dG undergoes oxidation by treatment with the transition metal oxidant \(\text{Na}_2\text{IrCl}_6\) \(^{62}\) \((E^\circ = 0.9\) V vs NHE\). However, unlike dOG and other C8-aryl dG adducts that undergo secondary oxidation to form mutagenic Sp and Gh lesions through \(\text{H}_2\text{O}\) attachment to the purine radical cation, treatment of \(p\)-PhOH-dG with \(\text{Na}_2\text{IrCl}_6\) or horseradish peroxidase (HRP)/\(\text{H}_2\text{O}_2\) generated C–C linked polymeric adducts through the intermediacy of the phenoxyl radical. \(^{62}\) This difference in reactivity was caused by conjugation of the phenolic ring in \(p\)-PhOH-dG with the purine radical cation leading to deprotonation of the phenol moiety and generation of the neutral phenolic radical to afford ortho–ortho \((o–o)\) C–C oligomeric cross-linked adducts (Figure 3-7). \(^{62}\) These studies demonstrated that \(p\)-PhOH-dG was susceptible to unique secondary oxidative processes and sparked interest into possible toxicological significance for this reactivity.

Chapter 3 investigates alternate phenol oxidation pathways and uses two radical-generating systems, \(\text{Cu}^{II}/\text{H}_2\text{O}_2\) \(^{64}\) and \(\text{Fe}^{II}\text{-EDTA/\text{H}_2\text{O}_2}\), \(^{12}\) to study the oxidative fate of \(p\)-PhOH-dG. The radical systems differ somewhat in that \(\text{Fe}^{II}\text{-EDTA/\text{H}_2\text{O}_2}\) generates freely diffusible \(\text{HO}^+\) while the \(\text{Cu}^{II}/\text{H}_2\text{O}_2\) system can generate a copper-oxo species that coordinates directly with DNA. \(\text{Fe}^{II}\text{-EDTA/\text{H}_2\text{O}_2}\) systems generate hydroxyl radicals in solution by classic Fenton chemistry (Equation 1). \(^{65,66}\) Studies investigating absolute efficiencies for radical attack to DNA molecules also found that \(\text{•OH}\) had the highest rate of attack for the ROS studied, followed by solubilised \(\text{e}^-\) and \(\text{H}^+\). \(^{67}\) This makes \(\text{•OH}\) the ROS of choice to study oxidative DNA interactions.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{•OH}
\]  

\(\text{(1)}\)
Copper is present in vivo at our molecule of interest (DNA), and products from interaction with p-PhOH-dG could be more biologically significant. For oxidation of dG by Cu\(^{II}/H_2O_2\), coordination of copper to N7 of dG is also thought to direct reactions in a more site specific manner.\(^6^4\) Copper is an important structural metal in chromatin, interacts with DNA at a rate of 1 per kilobase, and is involved in forming metalloprotein bridges which attach DNA to scaffold proteins.\(^6^8,6^9\) Furthermore, copper has been shown to play a critical role forming ROS in the vicinity of DNA.\(^6^5,7^0\) Our interest in this oxidation system stemmed from the known biological interaction between Cu and DNA. The Cu\(^{II}/H_2O_2\) system generates hydroxyl radical-like oxidizing agents, as shown in the equation 2.\(^7^0,7^1\)

\[
\begin{align*}
(1) \ Cu(II) + H_2O_2 & \rightarrow Cu(II) + H_2O + H^+ \\
(2) \ Cu(I) + H_2O_2 & \rightarrow Cu(II) + OH^- + \cdot OH
\end{align*}
\]

The goal of this chapter is to use different oxidation systems to study the fate of p-PhOH-dG in the presence of HO• given that phenolic toxins stimulate ROS production in biological systems and form C-linked adducts with dG. Furthermore, susceptibility of p-PhOH-dG to oxidative attack, as well as catechol and quinone production from phenol oxidation, stimulated interest in the possibility that quinone formation in DNA from secondary oxidation of a phenol adduct could play a significantly role in phenol toxicity. The results of these studies demonstrated that both systems facilitate hydroxylation of p-PhOH-dG to afford 3",4"-dihydroxyphenyl-dG (3",4"-DPh-dG) and show no propensity for mediating p-PhOH-dG polymerization through phenoxy radical generation.\(^6^2\) The product, 3",4"-DPh-dG, is more reactive than p-PhOH-dG and possesses a lower oxidation potential. This species also decomposed in basic aqueous media and reacted
covalently with thiol nucleophiles which strongly suggested the formation of the C8-quinone adduct (Figure 3-8).

**Figure 3 - 8:** Overall Scheme showing the potential oxidative fates of the p-PhOH-dG adduct addressed in the study. Oxidation of phenol produces the phenoxyl radical that can oxidize DNA through ROS (Path A). p-PhOH-dG is oxidized to form catechol adducts which undergo secondary oxidation to form quinones (Path B).
3.2. Experimental Procedures

3.2.1 General methods

A detailed description of the experimental procedures performed in Chapter 3 are given in Appendix A. Specific procedures are detailed below.

3.2.2 Synthesis of 3,4-bis-tert-butyldimethysiloxy(TBDMS)phenyl boronic acid, 8-(4'\'-hydroxyphenyl)-2'-deoxyguanosine (p-PhOH-dG) and 8-(3',4''-dihydroxyphenyl)-2'-deoxyguanosine (DHPh-dG).

3.2.2.1 Synthesis of 8-bromo-2'-deoxyguanosine.

**8-bromo-2'-deoxyguanosine (8-Br-dG).** 8-Br-dG was synthesized following well-established literature procedures.82 8.6 g (30.4 mmol) of dG and 400 mL of 4:1 CH$_3$CN:H$_2$O were added to a 1L Erlenmeyer flask with stirring. 7.7 g (43.1 mmol) of N-bromosuccinimide (NBS) was added slowly over 30 min and the reaction was stirred for 45 min at room temperature. The resulting suspension was filtered under vacuum, resuspended in 250 mL of cold acetone and stirred at room temperature for 2 h. The suspension was left at 0 °C overnight, filtered and washed with cold acetone. 8-Br-dG was collected as a faint pink solid (9.2 g, 90.5%); \^1H NMR (600 MHz, DMSO-d$_6$) δ 10.80 (s, 1H), 6.47 (s, 2H), 6.16 (t, J = 7.4 Hz, 1H), 5.23 (d, J = 4.5 Hz, 1H), 4.86 (t, J = 6.2 Hz, 1H), 4.39 (s, 1H), 3.80 (m, 1H), 3.60 (m, 1H), 3.51 (m, 1H), 3.15 (m, 1H), 2.11 (m, 1H); \^13C NMR (150 MHz, DMSO-d$_6$) δ 155.4, 153.1, 151.9, 120.5, 117.4, 87.8, 85.1, 71.1, 62.0, 36.5. HRMS (ESI) calcd for C$_{10}$H$_{12}$BrN$_5$O$_4$ 345.0073 found 345.0070 (MH\(^+\)).82
3.2.2.2. Synthesis of 3,4-bis-tert-butyl-dimethylsiloxy(TBDMS)phenyl boronic acid

Scheme 3 - 1: Synthesis of 3,4-bis-tert-butyl-dimethylsiloxy(TBDMS)phenyl boronic acid and 3′,4′-DPh-dG

3,4-bis-tert-butyl-dimethylsiloxy(TBDMS)phenyl boronic acid (1) was prepared as follows and shown in Scheme 3-1. Bromination of catechol was carried out by treating catechol with HBF$_4$ Et$_2$O at −30 °C in ACN followed by addition of 1 equiv N-bromosuccinimide and stirring for 17 h. Protection of the phenolic hydroxyl groups was achieved by treating the 4-bromocatechol with 3 equiv of tert-Butyldimethylsilyl chloride (TBDMSCI) in dry DMF in the presence of excess imidazole. Stirring for 2 h afforded the product. (1) was prepared by treating 3,4-bis-tert-dimethylsiloxybromophenyl with n-butyllithium and trimethyl borate at −78 °C in freshly distilled, dry DMF. All products were isolated in excellent yields (~ 80%) and purity (>90%). Pure products were confirmed by $^1$H, $^{13}$C NMR and HRMS and corresponded to known literature values.

3.2.2.3. Synthesis of p-PhOH-dG and DHPH-dG

8-(4′-hydroxyphenyl)-2′-deoxyguanosine (p-PhOH-dG). Suzuki-Miyaura cross-coupling of 8-Br-dG with phenol boronic acid was performed as previously reported. 0.129 mg (0.375 mmol) of 8-Br-dG, 14.8 mg (0.025 mmol) of TPPTS, 2.2 mg (0.01 mmol) of Pd(OAc)$_2$, 80 mg (0.75 mmol) of sodium carbonate and 156.3 mg (1.13 mmol)
of 4-hydroxyphenyl boronic acid were used to afford \( p\)-PhOH-dG as a light brown solid (79.4 mg, 78%); \(^1\)H NMR (600 MHz, DMSO-\( d_6 \)) \( \delta \) 10.70 (s, 1H), 9.90 (s, 1H), 7.44 (d, J = 8.6 Hz, 1H), 6.88 (d, J = 8.6 Hz, 1H), 6.34 (s, 2H), 6.03 (t, J = 6.7 Hz, 1H), 5.14 (m, 1H), 4.99 (m, 1H), 4.32 (m, 1H), 3.76 (m, 1H), 3.65 (m, 1H), 3.52 (m, 1H), 3.14 (m, 1H), 1.99 (m, 1H); \(^{13}\)C NMR (150 MHz, DMSO-\( d_6 \)) \( \delta \) 158.5, 156.6, 152.7, 151.8, 147.5, 130.6, 120.9, 116.8, 115.4, 87.8, 84.6, 71.2, 62.1, 36.5; HRMS (ESI) calcd for C\(_{16}\)H\(_{17}\)N\(_3\)O\(_5\) 359.1230 found 359.1224 (MH\(^+\)).

8-(3\(^\prime\),4\(^\prime\)-dihydroxyphenyl)-2\(^\prime\)-deoxyguanosine (3\(^\prime\),4\(^\prime\)-DHPH-dG). Scheme 3-2 outlines the synthesis of 3\(^\prime\),4\(^\prime\)-DHPH-dG from modified boronic acid. Suzuki–Miyaura cross-coupling of 1 with 8-Br-dG was performed according to the literature\(^{75}\) with slight modification due to solubility issues with the boronic acid. TBDMS-protected boronic acid 1 (420 mg, 1.11 mmol) was dissolved in 350 \( \mu \)L of CHCl\(_3\), and the solution was purged with argon for 30 min. This solution was then transferred to a 50 mL round-bottom flask containing 8-Br-dG (129 mg, 0.375 mmol), TPPTS (14.8 mg, 0.025 mmol), Na\(_2\)CO\(_3\) (80 mg, 0.75 mmol), and Pd(OAc)\(_2\) (2.2 mg, 0.01 mmol) in a solution of 1:1 water:methanol under argon. The reaction was heated to 95 °C and left to stir for 8 h. The reaction was diluted with 20 mL of water, and the pH was adjusted to \( \sim \) 7 with 10% HCl(aq). The mixture was cooled to 0 °C for several hours before the product was recovered by vacuum filtration. Under the basic conditions of the Suzuki–Miyaura cross-coupling conditions, the TBDMS protecting groups are removed.\(^{76,77}\) The final product 3\(^\prime\),4\(^\prime\)-DHPH-dG was obtained as a pale gray solid (0.083 g, 59%); \(^1\)H NMR (300 MHz, DMSO-\( d_6 \)) \( \delta \) 10.67 (bs, 1H), 9.33 (bs, 1H), 9.26 (bs, 1H), 6.99 (s, 1H), 6.85 (m, 2H), 6.29 (bs, 2H), 6.05 (t, J = 6.6 Hz, 1H), 5.11 (s, 1H), 4.97 (s, 1H), 4.31 (m, 1H), 3.76 (m, 1H), 3.63 (m, 1H), 3.52 (m, 1H), 3.13 (m, 1H), 1.96 (m, 1H); \(^{13}\)C NMR (75.5 MHz, DMSO-\( d_6 \)) \( \delta \)
156.6, 152.7, 151.6, 147.5, 146.8, 145.2, 121.2, 120.6, 116.8, 116.5, 115.4, 87.7, 84.3, 71.1, 62.1, 36.6; HRMS (ESI) calcd for C_{16}H_{17}N_{5}O_{6} 376.1173 found 376.1146 (MH^+).

3.2.3 Deglycosylation of p-PhOH-dG and 3"",4""-DHPH-dG.

Deglycosylation reactions of p-PhOH-dG and 3"",4""-DHPH-dG were achieved by placing the nucleosides (18 mg) in ca. 1 mL of 10% formic acid under heat (70 °C), as outlined previously.\textsuperscript{59,61,78} Authentic samples of p-PhOH-G and 3"",4""-DHPH-G were obtained following HPLC purification using 40 mM ammonium acetate, pH 7.2 (solvent A), and acetonitrile (solvent B) on a C-18 reverse-phase column with UV (280 nm) and fluorescence emission (390 nm) detection. The elution profile was a follows: 95/5 solvent A/B for 3 min followed by 7 min linear gradient to 90/10 A/B, followed by 20 min linear gradient to 70/30 A/B, and back to starting conditions (95/5 A/B) in 4 min. The product peaks were combined, lyophilized to dryness, and characterized by ESI-MS. These deglycosylated products along with the sample of 3"",4""-DHPH-dG were used to construct five-point HPLC calibration curves with UV detection to quantify levels produced from oxidation of p-PhOH-dG.

3.2.4 Oxidation Reactions.

3.2.4.1. Oxidation of p-PhOH-dG with Cu^{II}/H_{2}O_{2}.

A stock solution of p-PhOH-dG (0.01 M) was prepared in N,N-dimethylformamide (DMF). A solution of Cu(OAc)$_2$ (0.1 M) was prepared in 0.5 M aqueous PIPES buffer, pH 7.3. The reaction mixture consisted of 100 μL of p-PhOH-dG stock to 1 mL of PIPES buffer (0.5 M, pH 7.3). Aliquots (10 μL) of the Cu(OAc)$_2$ stock followed by 50 μL of H$_2$O$_2$.
(30%) were added. The resulting solution was stirred at 37 °C for various lengths of time and then quenched by the addition of 0.01 M EDTA. HPLC analysis of the reaction mixture was carried out using the protocol outlined for analysis of the deglycosylation reactions.

3.2.4.2. Oxidation of p-PhOH-dG with Fe⁺⁺-EDTA/H₂O₂.

Stock solutions of Fe(NH₄)₂(SO₄)₂ (0.01 M) and EDTA (0.02 M) were prepared in H₂O. A reaction mixture was prepared by adding 100 μL of the p-PhOH-dG DMF stock solution (0.01 M) to 1 mL of PIPES buffer (0.5 M, pH 7.3) containing 10 μL of both the Fe and the EDTA stock solutions. To this solution was added 50 μL of H₂O₂ (30%), and the reaction mixture was stirred at 37 °C for various lengths of time. Aliquots of the mixture were then analyzed by HPLC.

3.2.5 Basic Decomposition of 3ꞌꞌ,4ꞌꞌ-DHPh-dG in the Absence and Presence of NAC.

The basic aqueous decomposition of 3ꞌꞌ,4ꞌꞌ-DHPh-dG was followed spectrophotometrically by UV−vis and fluorescence emission (λex = 290 nm) spectroscopy at ambient temperature and by reverse-phase HPLC with UV detection. Reactions were initiated by adding 150 μL of a DMF stock solution of 3ꞌꞌ,4ꞌꞌ-DHPh-dG (0.01 M) to a 3 mL cuvette containing 1850 μL of 30% NH₄OH. Spectroscopic measurements were taken immediately after the addition of 3ꞌꞌ,4ꞌꞌ-DHPh-dG and following 30 s time intervals until no further change in the absorbance or emission spectra was observed (∼10 min). The solution was then concentrated under diminished pressure using a ThermoSavant DNA 120 SpeedVac at medium drying rate to remove
NH₄OH. The resulting neutral aqueous solution was characterized by ESI-MS. In separate experiments, the decomposition of 3",4"-DHP-dG in aqueous NH₄OH in the absence and presence of 50 equiv of NAC were monitored by reverse-phase HPLC. For these reactions, two solutions of 3",4"-DHP-dG were prepared by adding 100 μL of a stock solution in DMF (0.1 M) to 900 μL of water. To one solution was added NAC (81.6 mg, 0.050 mmol, 50 equiv) by a spatula, and the reaction mixture was stirred for ~2 min to dissolve NAC. To both solutions was added 1 mL of aqueous NH₄OH (10%) to initiate the reaction. Following various lengths of reaction time at ambient temperature, 100 μL aliquots were removed and neutralized with 0.1 M HCl to quench the reaction. The resulting neutral solutions were then analyzed by reverse-phase HPLC using an Agilent C-18 5 μ 150 mm × 4.6 mm column with a flow rate of 0.5 mL/min. For solutions lacking NAC, the following gradient conditions were employed: solvent A, aqueous 0.1% formic acid; solvent B, 70:30 ACN:aqueous 0.1% formic acid. The elution profile was as follows: 95/5 solvent A/B for 5 min followed by 5 min linear gradient to 90/10 A/B, followed by 15 min linear gradient to 70/30 A/B, and back to starting conditions (95/5 A/B) in 5 min. For solutions containing NAC, separation was carried out using buffer A [95:5 aqueous 50 mM triethylammonium acetate (TEAA), pH 7.2: ACN] and buffer B (70:30 ACN:50 mM TEAA). The elution profile was as follows: 95/5 solvent A/B for 5 min followed by 5 min linear gradient to 90/10 A/B, followed by 15 min linear gradient to 70/30, A/B, and back to starting conditions (95/5 A/B) in 5 min.
3.2.6. Cyclic Voltammetry (CV).

Electrochemical oxidation measurements were conducted in a three-electrode glass cell under N\textsubscript{2}. Measurements were carried out in a solution of 0.1 M DMF/tetrabutylammonium hexafluorophosphate. The working electrode was a glassy carbon electrode, 2 mm in diameter, polished and ultrasonically rinsed with ethanol. The reference electrode was an Ag wire placed in the same solution and separated from the main solution by a fine porosity frit. The reference electrode potential was calibrated in situ against 1 mol equiv of 9,10-anthraquinone [−0.800 V vs saturated calomel electrode (SCE)]. The counter electrode used was Pt wire wrapped in foil that was cleaned by repeated heating with a butane torch with cooling in pure Milli-Q water. For the CV, the starting potential was 0 V vs SCE, and the potential was first scanned 1.6 V toward positive potentials, and then scanned 1.6 V toward negative potentials. The scanning rate used was 0.2 V/s. Peak picking was achieved by manually shifting the CV values to correlate with the 9,10-anthraquinone internal standard and then using the Autolab software Gpes ver. 4.9.005 with data analysis method, peak picking feature, to determine peak potentials and half peak potentials.

3.2.7. Controlled Potential Electrolysis of p-PhOH-dG and 3′,4′-DHPPh-dG.

Electrochemical oxidation of p-PhOH-dG and 3′,4′-DHPPh-dG in the absence and presence of 25 equiv of NAC were carried out in a three-electrode glass cell under N\textsubscript{2} on a semi-preparative scale using controlled electrolysis. For the control experiments, stock solutions were made up with 0.01 mmol of the respective nucleoside (3.75 mg of 3′,4′-DHPPh-dG and 3.60 mg of p-PhOH-dG in 1 mL of DMF). The entire stock solution was added to 14 mL of a 150 mM phosphate buffer (pH 7.2), and the electrolysis was
initiated by applying the appropriate voltage (0.800 V for 3”,4”-DHPh-dG) and (1.100 V for p-PhOH-dG) for 90 min. The resulting solutions were analyzed by the HPLC method outlined below. For the experiments using NAC as a trapping agent, stock solutions of the modified bases were prepared in 1.0 mL of DMF/14 mL of phosphate buffer (pH 7.2), as outlined above, but 25 equiv of NAC (40.8 mg, 0.25 mmol) was added to the solutions prior to electrolysis. The potentials and electrolysis time were the same as the control experiments. The working electrode was a glassy carbon electrode, 15 mm in diameter, polished and ultrasonically rinsed with ethanol. The counter electrode was a Pt wire cleaned by repeated heating with a butane torch. The reference electrode was a Ag wire and was separated from the main solution by a fine porosity frit. The reactions were analyzed by HPLC using 40 mM ammonium acetate, pH 7.2 (solvent A), and 2:1 acetonitrile: 40 mM ammonium acetate (solvent B) with a semi-preparative C18 column with UV (280 nm) and fluorescence emission (390 nm) detection. The elution profile was as follows: 95/5 solvent A/B for 3 min followed by 7 min linear gradient to 90/10 A/B, followed by 17 min linear gradient to 70/30 A/B, and back to starting conditions (95/5 A/B) in 4 min. The NAC−conjugate peaks were combined, lyophilized, and characterized by UV−vis and ESI-MS.
3.3. Results and Discussion

3.3.1 Synthesis and characterization of p-PhOH-dG and DHPh-dG.

The two nucleosides used for this study were synthesized according to published literature procedures using the well established Suzuki-Miyaura coupling reaction\(^\text{62}\) to couple 8-Br-dG with the necessary boronic acid \(^\text{74}\) shown in Scheme 3-2. The 4-hydroxyphenyl boronic acid was available for purchase, but the 3,4-dihydroxyphenyl boronic acid was not available and needed to be synthesized. The procedure followed literature protocols and involved bromination of the catechol, silyl protection of the hydroxyl groups, and synthesis of the boronic acid (Scheme 3-1). Protection of the hydroxyl groups was necessary due to the pKa of hydroxyl groups on the boronic acid. The Suzuki-Miyaura procedure uses a base to activate the boronic acid. The pKa of catechol (\(\sim 8.7\))\(^\text{79}\) caused deprotonation of the hydroxyl groups during the reaction. Using the unprotected catechol resulted in failed reactions during initial coupling attempts. Upon addition of the silyl groups, however, solubility in the traditional ACN:H\(_2\)O solvent system was poor and modification was necessary. The boronic acid was dissolved in CH\(_3\)Cl first to obtain a solution under argon, and this solution was transferred to a second reaction vessel containing the remainder of the reagents in a 1:1 water:methanol solvent. Upon addition, some precipitation was observed, but heating the reaction aided with solubility. Deprotection of the catechol nucleoside occurred in the coupling reaction which required basic reaction conditions and further deprotection was unnecessary. The poor yield obtained for 3\(^\prime\),4\(^\prime\)-DHPh-dG (59\%) was attributed to limited solubility in the solvent system.
3.3.2. Hydroxyl Radical-Mediated Oxidation of p-PhOH-dG.

Phenols can undergo oxidative reactions to afford phenoxy radicals, semiquinone radicals, and quinone electrophiles that can react covalently with DNA to generate DNA adducts. Because dG is the most electron rich DNA base,\textsuperscript{15} it is particularly susceptible to covalent attachment by phenolic electrophiles\textsuperscript{7} and is prone to oxidation to generate electrophilic species that react with phenolate nucleophiles.\textsuperscript{30} This provides a diverse range of phenolic-dG adducts that may retain the dG and phenolic ring systems and are thus prone to further oxidative reactions. Furthermore, the
oxidation potentials for some dG adducts are lower than dG itself which promotes targeted secondary oxidation reactions.\textsuperscript{62}

This main focus of Chapter 3 examines secondary pathways generated through the oxidation of the nucleoside adduct $p$-PhOH-dG (Figure 3-8). This adduct is generated by mutagenic diazoquinones\textsuperscript{54} that break down to furnish aryl radical intermediates that undergo direct radical addition reactions at the C8-site of dG.\textsuperscript{80} Structurally similar C-linked phenolic adducts are also derived from sterically hindered phenoxy radical intermediates and the fate of these adducts, once formed, is critically important for determining mutagenic potency.

![Figure 3 - 9: UV-vis absorbance characteristics for A) $p$-PhOH-dG B) $p$-PhOH-G C) 3\textsuperscript{\prime\prime},4\textsuperscript{\prime\prime}$-DHPH-dG and D) 3\textsuperscript{\prime\prime},4\textsuperscript{\prime\prime}$-DHPH-G](image-url)
Treatment of $p$-PhOH-dG with the radical-generating systems produced species that eluted prior to the starting material (Figure 3-10). Incubation of the nucleoside $p$-PhOH-dG in the presence of Cu$^{II}$/H$_2$O$_2$ for 10 min generated a pair of peaks eluting at $\sim$25 min (Figure 3-10 Ai). After 30 min of incubation time (Figure 3-10 Aii), the product peaks were more prominent and showed distinct absorbances at 309 and 282 nm (Figures 3-9B and 3-9C), respectively. The retention time and spectroscopic properties of the product peaks suggested the presence of the hydroxylated species $3''$,$4''$-DHPh-dG (indicated by arrow in Figure 3-10 Ai) and the deglycosylated product $p$-PhOH-G that absorbs at 309 nm (Figure 3-9B).

In the presence of H$_2$O$_2$, Cu$^{II}$ has been proposed to generate hydroxyl radical-like species through Fenton type reactions$^{71}$ suggesting that the two products were produced by competing processes: (i) hydroxylation of the phenolic ring to generate $3''$,$4''$-DHPh-dG and (ii) H-atom abstraction from the sugar moiety to afford the deglycosylated base $p$-PhOH-G.

**Figure 3 - 10:** HPLC elution profiles from reaction of $p$-PhOH-dG with (A) Cu$^{II}$/H$_2$O$_2$ and (B) Fe$^{II}$-EDTA/H$_2$O$_2$ at 37 °C, pH 7.3, following (i) 10 min incubation, (ii) 30 min incubation, and (iii) 30 min incubation followed by treatment with acid (HCO$_2$H).
Figure 3-10B shows HPLC elution profiles from incubation of \( p \)-PhOH-dG in the presence of \( \text{Fe}^{II} \)-EDTA/\( \text{H}_2\text{O}_2 \). Following 10 min of incubation time (Figure 3-10Bi), \( p \)-PhOH-G and 3\(^\prime\prime\),4\(^\prime\prime\)-DHPh-dG were present, as noted in the \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) reaction. However, after 30 min of incubation (Figure 3-10Bii), the peak for \( p \)-PhOH-G was the dominant product peak. Also, a new peak, not observed in the \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) reaction, was noted following the 30 min incubation time that eluted at \( \sim \)23 min, exhibited an absorbance maximum of 319 nm and co-eluted with an authentic sample of the deglycosylated 3\(^\prime\prime\),4\(^\prime\prime\)-DHPh-G species (Figure 3-9D). No evidence for C\( \sim \)C linked polymeric adducts was found indicating that these systems did not facilitate phenoxyl radical production as previous one-electron oxidants had shown.\(^{62}\)

Following 30 min of incubation of \( p \)-PhOH-dG with \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) or \( \text{Fe}^{II} \)-EDTA/\( \text{H}_2\text{O}_2 \) in aqueous buffer, the catechol adduct 3\(^\prime\prime\),4\(^\prime\prime\)-DHPh-dG was produced by both systems (Figure 3-10). Hydroxylation of the phenolic ring system of \( p \)-PhOH-dG by \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) or \( \text{Fe}^{II} \)-EDTA/\( \text{H}_2\text{O}_2 \) is consistent with the known tendency of \( \text{HO}^\cdot \) to react with substituted benzenes by addition to the ring and not by interaction with the substituent.\(^{81}\)

**Table 3 - 1: Spectral Data for Oxidation of \( p \)-PhOH-dG\(^a\).**

<table>
<thead>
<tr>
<th>Adduct</th>
<th>( t_R )^( b )</th>
<th>( \lambda_{\text{max}} )^( c )</th>
<th>( [\text{M} - \text{H}]^{-} )</th>
<th>% yield(^ d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )-PhOH-dG</td>
<td>26.7</td>
<td>278</td>
<td>358.3</td>
<td>-</td>
</tr>
<tr>
<td>3(^\prime\prime),4(^\prime\prime)-DHPh-dG</td>
<td>24.9</td>
<td>280</td>
<td>374.1</td>
<td>20/3</td>
</tr>
<tr>
<td>( p )-PhOH-G</td>
<td>24.8</td>
<td>309</td>
<td>242.3</td>
<td>19/23</td>
</tr>
<tr>
<td>3,4-DHPh-G</td>
<td>22.8</td>
<td>319</td>
<td>258.2</td>
<td>-/11</td>
</tr>
</tbody>
</table>

\(^a\) A 10 mM \( p \)-PhOH-dG in 0.43 M PIPES buffer, pH 7.3, incubated at 37 °C for 30 min in the presence of \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) or \( \text{Fe}^{II} \)-EDTA/\( \text{H}_2\text{O}_2 \). \(^ b \) HPLC conditions: 95/5 solvent A (40 mM ammonium acetate pH 7.2)/B (acetonitrile) for 3 min followed by 7 min linear gradient to 90/10 A/B, followed by 20 min linear gradient to 70/30 A/B, and back to starting conditions (95/5 A/B) in 4 min. \(^ c \) In nm. \(^ d \) Yield for \( \text{Cu}^{II}/\text{Fe}^{II} \) reactions.
The oxidation reactions were also quenched with acid to deglycosylate all species in solution. The acidic solution was filtered, the precipitate was removed, redissolved in DMSO, and analyzed by HPLC. Following an initial incubation of \( p\)-PhOH-dG for 30 min in the presence of Cu\( ^{II}/H_2O_2 \) or Fe\( ^{II}/EDTA/H_2O_2 \), acid treatment produced the traces labeled (iii) in Figure 3-10. Both oxidation systems generated clean chromatograms featuring two major peaks that were identified as 3',4'-DHPh-G and \( p\)-PhOH-G on the basis of elution times, absorbance maxima, and mass. Spectral data for oxidation of \( p\)-PhOH-dG by the two radical generating systems (Cu\( ^{II}/H_2O_2 \) and Fe\( ^{II}/EDTA/H_2O_2 \)) are summarized in Table 3-1, and shown in Figure 3-9. Yields of 3',4'-DHP-dG, \( p\)-PhOH-G and 3',4'-DHPh-G were obtained from the 30 min incubation reaction which generated the highest yields due to further oxidative degradation of \( p\)-PhOH-dG upon longer incubation times. The yields were obtained through comparison to HPLC calibration curves using authentic adduct standards. Oxidation of \( p\)-PhOH-dG with Cu\( ^{II}/H_2O_2 \) generated more hydroxylated product (3',4'-DHPh-dG) than the Fe\( ^{II}/EDTA/H_2O_2 \) system while Fe\( ^{II}/EDTA/H_2O_2 \) yielded more deglycosylated \( p\)-PhOH-G product and generated 3',4'-DHPh-G, which was not observed in the Cu\( ^{II}/H_2O_2 \) system until treatment with acid (Figure 3-10 Aiii).

The relatively low yields of product are due to the presence of other product peaks that eluted earlier in the HPLC chromatogram and could not be identified due to the lack of authentic standards for comparison. Quenching the reactions in acid provided significant benefits for data analysis. In addition to stopping the reaction at a defined time, deglycosylating the bases separated the peaks in the HPLC traces which is most apparent in Figure 3-10iii. This allowed accurate quantification to be performed on products and reliable comparisons of reaction products to be made.
Rate constants for hydroxylation of phenol by \( \text{HO}^- \) are large \((10^7\text{--}10^9 \text{ M} \cdot \text{s}^{-1})\) with the electrophilic \( \text{HO}^- \) showing a strong preference for attack at the ring positions activated by the phenolic \( \text{HO} \) group (\( o \) and \( p \)). Given that the \( p \)-position of \( p\text{-PhOH-dG} \) is blocked by the \( \text{dG} \) moiety, \( o \)-hydroxylation to afford \( 3''\text{-}4''\text{-DHPH-dG} \) was proposed as the probable reaction pathway. This is in agreement with several studies on hydroxylation of substituted phenols which show \( \cdot\text{OH} \) attack at the three possible positions gives a ratio of \( \text{OH} \) attachment to one ring position, \( p\text{(para)}:\text{p(ortho)}:\text{p(meta)} \) is 9:6:1 (Figure 3-11). 81

Figure 3 - 11: Oxidative pathways for phenol. Phenol can be deprotonated to the phenolate anion and the phenoxy radical which can dimerize or undergo hydroxylation on the phenyl ring.

Both systems caused deglycosylation of \( p\text{-PhOH-dG} \) to generate \( p\text{-PhOH-G} \); the \( \text{Fe}^{II}\text{-EDTA/H}_2\text{O}_2 \) system also produced \( 3''\text{-}4''\text{-DHPH-G} \). These findings suggested that \( \text{H} \) atom abstraction from the sugar moiety to afford the deglycosylated base \( p\text{-PhOH-G} \) competes with hydroxylation of the phenolic ring to generate \( 3''\text{-}4''\text{-DHPH-dG} \). The ratios of \( 3''\text{-}4''\text{-DHPH-dG} \) to \( p\text{-PhOH-G} \) were \( \sim 1 \) for \( \text{Cu}^{II}/\text{H}_2\text{O}_2 \) and \( \sim 0.13 \) for \( \text{Fe}^{II}\text{-EDTA/H}_2\text{O}_2 \).
(Table 3.1). The ratio determined for the Fe$^{II}$-EDTA/H$_2$O$_2$ system does not take into account the production of 3\''\',4\''\'-DHPh-G, because its mode of formation is uncertain. This species could have arisen from deglycosylation of 3\''\',4\''\'-DHPh-dG or hydroxylation of p-PhOH-G. That the Fe$^{II}$-EDTA/H$_2$O$_2$ system generated more deglycosylated product p-PhOH-G correlated somewhat with findings by the Dedon laboratory on levels of dOG and strand breaks produced by Cu$^{II}$/H$_2$O$_2$ vs Fe$^{II}$-EDTA/H$_2$O$_2$ in reaction with duplex DNA.\textsuperscript{64} Ratios of dOG to strand breaks were $\sim$0.4 for Cu$^{II}$/H$_2$O$_2$ and $\sim$0.03 for Fe$^{II}$-EDTA/H$_2$O$_2$ demonstrating that the Fe$^{II}$ system is more efficient at generating strand breaks through H-atom abstraction from the sugar moiety.\textsuperscript{64}

Dedon and colleagues observed similar trends with differing oxidants and attributed the proximity of the oxidizing agents to the different sites of hydrogen abstraction to explain their observations. Both Cu(I) and Cu(II) are known to associate with the N7 of guanine.\textsuperscript{64} Dedon postulated that generation of a different DNA-damaging species by either oxidation strategy or DNA binding specificity of Cu(II) might account for the difference in hydrogen abstraction preference.

Both oxidation systems produce hydroxyl radical-like oxidants and we propose that coordination of Cu(II) with guanine is influential in producing more hydroxylated products. Preferential binding of Cu(II) to the N7 of guanine causes the C8 position to be susceptible to attack by a hydroxyl radical or some other Cu-induced oxidizing species. For this study, production of hydroxyl radicals in close proximity to the phenol moiety suggested a preference for hydroxylation on the phenyl ring in comparison to hydrogen abstraction from the sugar.

Finally, initial studies on the oxidative fate of p-PhOH-dG performed by Weishar and colleagues, employed the transition metal oxidant Na$_2$IrCl$_6$ that was previously
utilized by the Burrows laboratory to study the oxidation of dOG.\textsuperscript{53} In the presence of Na\textsubscript{2}IrCl\textsubscript{6}, \( p \)-PhOH-dG was converted into a neutral phenoxy radical that underwent coupling reactions to afford polymeric adducts (Figure 3-7). HPLC-MS analysis showed the presence of three polymeric adducts (dimer, trimer, and tetramer) that eluted after the starting material \( p \)-PhOH-dG.\textsuperscript{62} Treatment of \( p \)-PhOH-dG with HRP/H\textsubscript{2}O\textsubscript{2} also generated the C-C-coupled polyphenols through the intermediacy of the phenoxy radical. Interestingly, the two oxidation systems used in current experiments did not produce any polymers and instead gave rise to hydroxylation reactions, suggesting that oxidation pathways taken by \( p \)-PhOH-dG are oxidant specific. The secondary oxidative pathway for phenolic C8 adducts produced from these studies had not previously been observed and was hypothesized to significantly impact toxicity of phenolic adducts given the properties and reactivity of catechol species.\textsuperscript{82}

3.3.3. Properties and reactivity of 3\textsuperscript{″},4\textsuperscript{″}-DHP-dG.

Having established that treatment of the parent \( p \)-PhOH-dG adduct with Cu\textsuperscript{II}/H\textsubscript{2}O\textsubscript{2} or Fe\textsuperscript{II}-EDTA/H\textsubscript{2}O\textsubscript{2} yields the hydroxylated adduct 3\textsuperscript{″},4\textsuperscript{″}-DHP-dG, its properties and reactivity were determined. The optical properties of 3\textsuperscript{″},4\textsuperscript{″}-DHP-dG in aqueous media at pH 7 and its electrochemical redox properties in DMF were characterized in order to draw comparison to the corresponding properties of \( p \)-PhOH-dG.\textsuperscript{60,78,83} Given that other catechols are unstable in aqueous basic media and undergo conversion to semiquinone radicals and quinones by an auto-oxidative process,\textsuperscript{46,50,84} the reactivity of 3\textsuperscript{″},4\textsuperscript{″}-DHP-dG in aqueous NH\textsubscript{4}OH was studied in the absence and presence of the thiol nucleophile \( N \)-acetylcysteine (NAC), which was added as a trapping agent. These experiments were then compared to the reactivity of 3\textsuperscript{″},4\textsuperscript{″}-DHP-
dG in aqueous buffered media pH 7.2 following electrochemical oxidation in the absence and presence of excess NAC.

### 3.3.3.1. Optical and redox properties of 3″,4″-DHPH-dG.

The optical and redox properties of 3″,4″-DHPH-dG are listed in Table 3-2. Absorption and emission spectra were recorded in aqueous MOPS buffer, pH 7.0. Optical data for unmodified dG\textsuperscript{51} and p-PhOH-dG\textsuperscript{60,62} in aqueous media are included for comparison. As discussed previously, attachment of the phenolic moiety to the C8-site of dG to afford the nucleoside p-PhOH-dG causes a red-shift in absorption maxima (278 vs. 253 nm) and generates a highly fluorescent nucleobase ($\lambda_{\text{em}} = 390$ nm, $\Phi_{\text{fl}} = 0.47$) that acts as a pH-sensitive fluorescent probe.\textsuperscript{60} Addition of a second auxochromic OH to generate 3″,4″-DHPH-dG increases the red shift (282 vs 278 nm) in absorbance and fluorescence; however, the intensity of the fluorescent signal was decreased compared to p-PhOH-dG ($\lambda_{\text{em}} = 392$ nm, $\Phi_{\text{fl}} = 0.15$, Table 3-2).

**Table 3 - 2: Photophysical and Redox Parameters for Phenolic C-linked Adducts**

<table>
<thead>
<tr>
<th>Adduct</th>
<th>$\lambda_{\text{max}}$, log $\varepsilon$</th>
<th>$\lambda_{\text{em}}$ ($\Phi_{\text{fl}}$)</th>
<th>$E_{\text{p/2}}$ (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-PhOH-dG</td>
<td>278, 4.26</td>
<td>390 (0.47)</td>
<td>0.85</td>
</tr>
<tr>
<td>3″,4″-DHPH-dG</td>
<td>282, 4.37</td>
<td>392 (0.15)</td>
<td>0.71</td>
</tr>
<tr>
<td>dG\textsuperscript{a}</td>
<td>253, 4.14</td>
<td>334 (9.7 x 10\textsuperscript{-5})</td>
<td>1.14</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Optical data for dG taken from ref \textsuperscript{51}.\textsuperscript{b} Determinded in aqueous 10 mM MOPS buffer, pH 7.0, $\mu = 0.1$ M NaCl.\textsuperscript{c} Determined using the comparative method with quinine bisulfate in 0.5 M H\textsubscript{2}SO\textsubscript{4} ($\Phi_{\text{fl}} = 0.55$).\textsuperscript{d} Half-peak potentials ($E_{\text{p/2}}$) in volts (V) vs SCE using CV in anhydrous DMF with a glassy carbon working electrode.
The electron-donor properties of 3",4"-DPh-dG were determined using cyclic voltammetry (CV) in anhydrous DMF as outlined previously for other nucleoside adducts.\textsuperscript{60,62,83} Under these experimental conditions, the catechol nucleoside 3",4"-DPh-dG showed an irreversible 1\textsuperscript{e} oxidation peak with a half-peak potential ($E_{p/2}$) of 0.71 V vs. SCE which is 0.14 V lower than the corresponding value for $p$-PhOH-dG (0.85 V/SCE) and 0.43 V lower than the value obtained for dG ($E_{p/2} = 1.14$ V/SCE, Table 3-2).

Adding an OH group lowered the oxidation potential in DMF (Table 3-2), indicating that 3",4"-DPh-dG is a better electron donor than $p$-PhOH-dG. This is expected as each oxygen atom for the phenolic OH group contributes two $\pi$-electrons to conjugation within the ring system, making it electron rich.\textsuperscript{85}

The lowered oxidation potential was found to have important consequences in terms of reactivity. Treatment of $p$-PhOH-dG with aqueous NH$_4$OH favors dianion formation as $p$-PhOH-dG has two acidic protons (phenolic OH and N1) with $pK_a$ values ~ 9.\textsuperscript{60} In NH$_4$OH the adduct showed an absorption band at ~ 305 nm that remained stable over a 2 h period at ambient temperature (Figure 3-15). In contrast, 3",4"-DPh-dG was sensitive to aqueous NH$_4$OH and decomposed to a stable product (96% yield) following incubation at ambient temperature (Figure 3-13). Product analysis by ESI$^-$-MS (Figure 3-14) was consistent with formation of a dicarboxylic acid derivative.
Figure 3-12: Proposed Pathway for the Aqueous Decomposition of 3″,4″-DHPPh-dG showing the formation of trianion and subsequent formation of the quinone. Quenching with acid forms the dicarboxylic acid.

Diacids are known to be generated from o-quinones by reaction with HOO⁻ that initiates C-C cleavage via an acyclic Baeyer-Villiger type mechanism.⁸⁶ Outlined in Figure 3-12 is a proposal for diacid formation that highlights o-quinone formation and HOO⁻ production from treatment of 3″,4″-DHPPh-dG with NH₄OH in the presence of molecular oxygen (O₂). Deprotonation of 3″,4″-DHPPh-dG by NH₄OH will generate anionic species and the dianion from deprotonation of N1 and phenolic OH para to the dG component is shown in equilibrium with the trianion resulting from deprotonation of N1 and both phenolic OH groups. The initiating step in the decomposition process involves transfer of one electron from the anionic species to O₂ to form O₂⁻ and the semiquinone radical anion;⁸⁷,⁸⁸,⁸² depicted in Figure 3-12. In turn, O₂⁻ reacts with the anion species to generate another equivalent of the semiquinone radical anion and H₂O₂ (HOO⁻ in base).⁸² Disproportionation of the transient semiquinone radical anion generates the transient o-quinone anion and anionic species of 3″,4″-DHPPh-dG.⁸²,⁸⁹
Reaction of the o-quinone species with HOO⁻ generates the neutral diacid product following acidification and isolation (Figure 3-12).

![UV-vis spectral changes](image)

**Figure 3 - 13:** (A) UV-vis spectral changes during the aqueous NH₄OH (30%) decomposition of 3″,4″-DPh-dG at ambient temperature. Absorption measurements were taken immediately after addition of 3″,4″-DPh-dG to 30% NH₄OH and following 30 s time intervals until no further change in absorbance was observed (~ 10 min). (B) HPLC elution profile of the aqueous NH₄OH (10%) decomposition of 3″,4″-DPh-dG: (i) before NH₄OH treatment, and following treatment for (ii) 10 min, (iii) 30 min, and (iv) 120 min at ambient temperature.

### 3.3.3.2. Basic decomposition of 3″,4″-DPh-dG in the absence and presence of NAC.

Spectral changes (UV-vis and fluorescence emission) of 3″,4″-DPh-dG in aqueous NH₄OH (30%) at ambient temperature were initially monitored to provide information on the stability of 3″,4″-DPh-dG in basic aqueous solution. The neutral species absorbs at ~ 282 nm and shows emission at ~ 392 nm (Table 3-2). However, in 30% NH₄OH the initial absorption spectrum (Figure 3-13A) showed a broad absorption band at ~ 435 nm that decreased in intensity and was no longer visible after 5 min. After 10 min no further changes in the collected spectra were noted and a product with a maximum peak at 280 nm and a prominent shoulder at 330 nm were present. Over the
same time period the fluorescence emission intensity ($\lambda_{ex} = 290$ nm) of the basic solution increased and shifted from $400$ nm to $390$ nm (Figure 3-15B).

Figure 3 - 14: ESI-MS analysis of product from the aqueous NH$_4$OH (30%) decomposition of 3″,4″-DHPh-dG. (A) ESI$^-$-MS spectrum with $[M - H]^-$ = 406. (B) MS$^3$ spectrum taken at $m/z$ 290.

Under analogous conditions, $p$-PhOH-dG showed an absorption band at $\sim 305$ nm that did not change over a 2 h time period (Figure 3-15A). The absorption at 305 nm was ascribed to the dianion of $p$-PhOH-dG from deprotonation of the phenolic OH group and N1 of the dG component which have $pK_a$ values $\sim 9$. These results suggested that deprotonation of $p$-PhOH-dG to form the dianion occurs rapidly and the product is stable in basic aqueous solution while the anionic species of 3″,4″-DHPh-dG decomposes.
Figure 3 - 15: A) UV-vis spectra of ρ-PhOH-dG in aqueous 30% NH₄OH taken immediately after addition of the nucleoside to NH₄OH (dotted trace) and following 2 h incubation (solid trace). B) Fluorescence emission (λ<sub>ex</sub> = 290 nm) spectral changes during the aqueous NH₄OH (30%) decomposition of 3″,4″-DPh-dG. Spectroscopic measurements were taken immediately after addition of 3″,4″-DPh-dG to 30% NH₄OH and following 30 s time intervals until no further change in the absorbance or emission spectra were observed (~ 10 min).

Further information on the aqueous decomposition of 3″,4″-DPh-dG was obtained by HPLC analysis of the reaction mixture (Figure 3-13B). For HPLC analysis, the decomposition of 3″,4″-DPh-dG was carried out in 10% NH₄OH. Following 2 h incubation at ambient temperature, 3″,4″-DPh-dG had completely decomposed (Figure 3-13Biv) and a single major product (96% yield, based on HPLC peak integration assuming similar λ = 280 of the product and starting material) eluting at ~ 5 min was observed. This product peak had λ<sub>max</sub> at 280 nm and a prominent shoulder at 330 nm (from diode array analysis) as observed in the UV spectra recorded for the decomposition of 3″,4″-DPh-dG in 30% NH₄OH following 10 min incubation at ambient temperature (Figure 3-13A).
The product from the basic aqueous decomposition of 3",4"-DHPh-dG was characterized by ESI-MS. The ESI\(^{-}\)-MS analysis showed a parent ion with [M – H]\(^-\) = 406 (Figure 3-14A) for a neutral mass m/z 407; 32 mass units heavier than 3",4"-DHPh-dG. A fragment ion at m/z 290, for loss of the deoxyribose (-116) moiety, was observed, and this species showed fragment ions at m/z 273 and 247 (Figure 3-14B) for nominal mass losses of 17 and 43, respectively. These losses are also observed for deprotonated p-PhOH-G,\(^b\) and suggest loss of NH\(_3\) (17) and isocyanic acid (HNCO, 43) from the guanine component of the product.

![Diagram](image)

**Figure 3 - 16:** ESI\(^{-}\)-MS analysis of proposed diacid product from the aqueous NH\(_4\)OH (30%) decomposition of 3",4"-DHPh-dG. (A) ESI\(^{-}\)-MS spectrum with [M + H]\(^+\) = 408 and major fragment at m/z 292 for loss of deoxyribose (-116). (B) MS\(^3\) spectrum taken at m/z 292, (C) MS\(^4\) spectrum taken at m/z 275, (D) MS\(^5\) spectrum taken at m/z 258, and (E) MS\(^6\) spectrum taken at m/z 230.

The ESI\(^{-}\)-MS analysis of the neutral solution (Figure 3-16A) turned out to be more diagnostic of the product generated from the aqueous decomposition of 3",4"-DHPh-dG. Here, both the MS\(^3\) (Figure 3-16B) and MS\(^4\) (Figure 3-16C) spectra of the decomposition product showed single fragments at m/z 275 and 258 for losses of 17
mass units. The MS\textsuperscript{5} (Figure 3-16D) and MS\textsuperscript{6} (Figure 3-16E) spectra gave fragment ions at \textit{m/z} 230 and 202, respectively, for losses of 28 mass units. These fragment ions (loss of 17 mass units (OH) followed by loss of 28 mass units (CO)) are indicative of carboxylic acid groups and suggested that 3\textasciitilde,4\textasciitilde-DHPh-dG decomposed into the diacid shown in Figure 3-16 that has a neutral mass of \textit{m/z} 407. As described by Sawaki and Foote,\textsuperscript{86} o-quinones undergo C-C cleavages through reaction with HOO\textsuperscript{−} via an acyclic Baeyer-Villiger type mechanism to afford dicarboxylic acid derivatives. In the absence of added H\textsubscript{2}O\textsubscript{2} to the basic solution, electron-transfer from the phenolate to O\textsubscript{2} generates O\textsubscript{2}\textsuperscript{−} and the semiquinone radical.\textsuperscript{87,88} This process initiates \textit{in situ} H\textsubscript{2}O\textsubscript{2} production (HOO\textsuperscript{−} in base) and the C-C cleavage in the base-catalyzed autoxidation of catechols also proceeds via the acyclic mechanism involving an o-quinone intermediate.\textsuperscript{86}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{HPLC elution profile of the aqueous NH\textsubscript{4}OH (10\%) decomposition of 3\textasciitilde,4\textasciitilde-DHPh-dG in the presence of 50 equiv NAC: (i) initial 3\textasciitilde,4\textasciitilde-DHPh-dG and following treatment for (ii) 30 min, (iii) 4 h, and (iv) 24 h at ambient temperature.}
\end{figure}
The basic decomposition of 3"',4"'-DHPh-dG was also carried out in the presence of 50 equiv NAC in an effort to trap the expected α-quinone intermediate since α-quinones are well known to react with thiols by conjugate addition reactions. HPLC analysis of the reaction mixture (Figure 3-17) indicated that NAC decreased the rate of 3"',4"'-DHPh-dG decomposition considerably. Following 24 h incubation (Figure 3-17iv) at ambient temperature ~ 10% 3"',4"'-DHPh-dG was still present (following 2 h incubation in the absence of NAC, 3"',4"'-DHPh-dG had totally decomposed, Figure 3-17iv).

The major product of this reaction was the diacid (~ 78%), although a new species was present (~ 11%) eluting just before the starting material (Figure 3-17iv, labeled with the asterisk). ESI-MS analysis of this product gave a parent ion at [M – H]− = 535 (Figure 3-18) for attachment of NAC to 3"',4"'-DHPh-dG followed by the loss of 2H (i.e. 375 (3"',4"'-DHPh-dG) + 163 (NAC) – 2H = 536). The fragment ion at m/z 406 is 32 mass units heavier than 3"',4"'-DHPh-dG and results from β-elimination of benzoquinol-SH which is an established fragmentation of S-peptide-benzoquinone adducts. The insert in Figure 3-18 shows the MS3 spectrum taken at m/z 406 showing loss of the
deoxyribose moiety (-116 mass units). These results provided further support for the notion that the aqueous decomposition of 3″,4″-DHPh-dG generates an electrophilic α-quinone intermediate.

3.3.3.3 Controlled potential electrolysis.

To compare the reactivity of p-PhOH-dG to 3″,4″-DHPh-dG in aqueous media at neutral pH in the absence of O₂/H₂O₂, their electrochemical oxidation in the absence and presence of 25 equiv NAC was examined. These experiments were carried out in aqueous phosphate buffer (pH 7.2) under N₂ on a semipreparative scale using controlled electrolysis. With no NAC trapping agent, the p-PhOH-dG solution turned a faint yellow color upon electrolysis and a thick black shiny coating developed on the working electrode surface that could only be removed by vigorous polishing methods.

![Figure 3-19: HPLC elution profile from electrochemical oxidation of 3″,4″-DHPh-dG in the presence of 25 equiv NAC.](image)

A) 3″,4″-DHPh-dG

Retention time (min)

- + 2 NAC
- + 1 NAC

B) ((NAC)_2-3″,4″-DHPh-dG [M - H]^+ = 696 )
HPLC analysis of the electrolysis mixture showed no new peaks when compared to the initial chromatogram (not shown). In the presence of 25 equiv NAC, the solution again turned a faint yellow color upon electrolysis and the thick coating was observed on the electrode surface. HPLC analysis again showed no new peaks from the electrolysis procedure. In both electrolysis experiments, the starting material was diminished after electrolysis although no new peaks were observed by HPLC. These results suggested polymerization of $p$-PhOH-dG through phenoxy radical production\textsuperscript{62} to generate an insoluble polymer that coated the electrode surface.\textsuperscript{94}

![Figure 3 - 20: UV-vis spectra of (A) 0.19 mM NAC-3″,4″-DHP-dG and (B) 0.14 mM (NAC)$_2$-3″,4″-DHP-dG in aqueous 50 mM phosphate buffer, pH 7.2, at room temperature](image)

Electrolysis of 3″,4″-DHP-dG in the absence of NAC generated a yellow solution and a dark coating on the electrode surface. Analysis of the electrolysis solution by HPLC indicated that the starting material had diminished although no new peaks were observed, as noted for electrolysis of $p$-PhOH-dG. In the presence of 25 equiv NAC, the solution again turned yellow and the dark coating was observed on the electrode surface. However, two new peaks were observed by HPLC, eluting faster than the starting material (Figure 3-19). The UV-vis spectrum of the species giving rise to the peak labeled +1 NAC (Figure 3-19A) had $\lambda_{\text{max}}$ 261 nm and a broad shoulder peaking at
283 nm (Figure 3-20A) that matched the diode array spectrum for NAC-3″,4″-DHPH-dG generated from the aqueous 10% NH₄OH decomposition of 3″,4″-DHPH-dG in the presence of 50 equiv NAC. ESI⁻-MS analysis confirmed the presence of the mono-NAC conjugate with an [M – H]⁻ ion at 535 (Figure 3-18). Isolation of the species giving rise to the peak labeled +2 NAC (Figure 3-19) generated a UV-vis spectrum with absorbances at 320 and 272 nm (Figure 3-20B). The red shift from the second NAC addition is characteristic of glutathione adducts with similar structures and is attributed to addition of an auxochromic sulphur molecule to the chromophore.

Figure 3 - 21: A) ESI⁻-MS spectrum of peak +2 NAC. B) MS⁴ taken at m/z 438 showing loss of sugar (-116).

ESI⁻-MS analysis of the product showed an [M – H]⁻ ion at 696 with fragment ions at m/z 567 and 438 for attachment and subsequent cleavage of the S-C bond¹ of two NAC groups (Figure 3-21A). Figure 3-21B shows the MS⁴ spectrum taken at m/z 438 that displays a dominant fragment at m/z 322 corresponding to loss of deoxyribose (-116). These observations were consistent with formation of a di-NAC conjugate ((NAC)₂-3″,4″-DHPH-dG). The potentials employed for electrolysis were slightly above the 1e⁻ oxidation potentials (Table 3-2) of the nucleoside adducts to target oxidation.
reactions to the species of interest. 3",4"-DHP-dG was converted into the semiquinone radical and p-PhOH-dG formed the phenoxy radical. Under these electrolysis conditions, p-PhOH-dG appeared to form a polymer on the electrode surface, as polymerization of phenols is known to generate an insulating film that sticks to the electrode,\textsuperscript{94} and failed to react with NAC. 3",4"-DHP-dG also appeared to form a polymer on the electrode surface in the absence of NAC but reacted with NAC to form mono-NAC and di-NAC conjugates (Figure 3-19).

Formation of di-GSH conjugates is common for reactions of o-quinones with GSH indicating that the mono-conjugates are very good substrates for further conjugation reactions.\textsuperscript{92} Attack from the nucleophile to the double bond of the quinone produces the reduced catechol conjugate. This species has shown excellent candidacy for further oxidation and is oxidized a second time to form the conjugate quinone.\textsuperscript{95} Secondary nucleophilic attack from the sulphur trapping agent on the mono conjugated quinone forms the di-NAC-conjugate observed in Figure 3-19.

![Chemical Structure](image)

**Figure 3 - 22:** Oxidation of BQ-dG to form hydroxylated BQ-dG adducts if oxidation occurs on the phenyl ring and 8-oxo-BQ-dG adducts if oxidation occurs on guanine.\textsuperscript{94}

These observations are consistent with known phenolic radical reactivity to undergo polymerization reactions\textsuperscript{99} while semiquinone radicals reactivity can also
proceed via disproportionation to afford o-quinones that react covalently with thiol nucleophiles.\textsuperscript{89,91,92}

That electrolysis of 3″,4″-DHP-dG in the presence of excess NAC generated the di-NAC conjugate (NAC)\textsubscript{2}-3″,4″-DHP-dG (Figure 3-19), correlates well with known o-quinone electrophile reactivity with GSH to generate di-GSH conjugates. Furthermore, the electrophile \textit{p}-benzoquinone (BQ) is known to react with dG to afford a benzetheno adduct that contains the phenolic and dG ring systems (Figure 3-22).\textsuperscript{96} Gaskell and coworkers have reported that the BQ-dG adduct undergoes hydroxylation of the phenolic ring to afford a catechol adduct.\textsuperscript{97} More recent results by Linhart et al. show that benzetheno adducts are extensively metabolized \textit{in vivo}.\textsuperscript{55} Thus, as outlined in Figure 3-22, BQ-dG undergoes hydroxylation at the phenolic ring which competes with hydroxylation at the C8-site of BQ-dG to afford BQ-dOG \textit{in vivo}.\textsuperscript{55} Secondary oxidative pathways of oxidized adducts are important for their mutagenicity, as noted for oxidation of dOG.\textsuperscript{21,27} Quinones are known to form covalent adducts with nucleobases and production of a quinone from secondary oxidation of the \textit{p}-PhOH-dG adduct in DNA is predicted to react, forming cross-links. The creation of site-specific DNA cross-links through o-quinone formation may have greater toxicological importance, as these lesions are more difficult to repair.\textsuperscript{98}

3.4. Conclusions

The results of our studies revealed the following; \textit{p}-PhOH-dG is oxidized by hydroxyl radical generating systems to produce 3″,4″-DHP-dG. This alternate oxidation pathway had not been previously observed \textit{in vitro} and competes with hydrogen
abstraction from the sugar moiety to produce deglycosylation. The quantity of 3″,4″-DPh-dG observed differed between the two ROS generating systems and is suggestive of proximity effects from hydroxyl radical generation. 3″,4″-DPh-dG has a lowered oxidation potential which makes it a clear target for further oxidative reactions. Furthermore, like many catechols, it appeared to undergo secondary oxidative reactions to form a quinone that was trapped with NAC. Production of quinones within DNA, places the lesion in close proximity to many nucleophiles that have been shown to produce DNA adducts. All of the results suggest that secondary oxidative pathways of dG lesions bearing phenolic rings could influence toxic effects observed for C8 phenol adducts.

Future studies should focus on incorporating the catechol adduct into DNA strands to determine the structural and oxidative consequences of this adduct. It will be quite interesting to observe how the DNA environment influences the reactivity of the phenoxy radical and the o-quinone electrophile, given that o-quinones typically undergo conjugate addition to N7 of dG, while the phenoxy radical undergoes C-C coupling which may be impossible within duplex DNA.

3.5. References


(2) Reiter, R. J., Faseb J., 1995, 9, 526-533.


Chapter 4.

Functionalization of Thrombin Binding Aptamer by 3-Furyl Modification for Targeted Covalent Binding of Thrombin Protein
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4.1. Introduction

Nucleic acids are often solely thought of as the template for life, which codes for successful reproduction, and contributes to species diversification. However, for approximately as long as these template functions have been studied, nucleic acids have been known to perform a wide array of alternate biochemical functions, fueled by the inherent diversity in three-dimensional folding capability.\textsuperscript{1-3} As early as the 1980's, it was known that RNA could catalyze chemical reactions. These functionalized nucleic acids were called ribozymes.\textsuperscript{4} Synthetic ribozyme molecules have shown the ability to carry out an array of chemical reactions from phosphodiester bond formation to Diels Alder reactions.\textsuperscript{2}

With revelations concerning secondary capabilities of nucleic acids, and the rapid development of synthetic methods capable of creating large libraries of random DNA and RNA sequences, functionalized oligonucleotide research is an exciting and rapidly growing field. DNA polymers adopt different secondary conformations depending on the degree of hydration, salt content, or base-pairing sequence.\textsuperscript{5} These alternate structures play various roles in gene regulation,\textsuperscript{6} genetic stability, and cellular senescence.\textsuperscript{7} Telomeres, for example, adopt alternate quadruplex structures based on their G-rich sequence to help stabilize chromosomes.\textsuperscript{8} Conformational flexibility possessed by nucleic acids not only diversifies their natural uses in cells but makes them attractive candidates for small molecule binding research.\textsuperscript{9}

The term aptamer was originally proposed by Ellington and Szostak\textsuperscript{10} and was used to describe single stranded oligonucleotides, both RNA and DNA, that could bind specific targets with high affinity due to the wide array of possible three-dimensional motifs adopted by oligonucleotides.\textsuperscript{10} Aptamers are chosen from a large library of
different sequences by passing them through a column or allowing them to incubate with a bound target. After eluting the sequences that have some affinity for the target, they are amplified by polymerase chain reaction (PCR), and this condensed library is once again screened with the desired target by increasing the stringency of the selection process. The entire operation is referred to as systematic evolution of ligands by exponential amplification (SELEX) and is depicted in Figure 4-1. Following the selection process, a few sequences are obtained from the original library of perhaps $10^{15}$ sequences that bind the desired target with very high affinity.

Figure 4 - 1: SELEX selection process for screening aptamer libraries.

Aptamers for protein targets arose from the examination of viral RNA and the close associations these oligonucleotides demonstrated for specific viral proteins. A review by Cullen and Greene notes that certain viral gene transcripts formed close associations with proteins, regulating their expression time to evade immune detection.
This process was essential for their successful replication.\textsuperscript{12} Subsequent evaluation regarding RNA aptamer ability to inhibit pathological protein targets produced some excellent successes.\textsuperscript{13} Further support for the candidacy of aptamers for protein binding came from findings that they met or exceeded most comparisons to antibodies, another specific protein binder.\textsuperscript{14} A list of the full comparisons between aptamers and antibodies is shown in Table 4-1. Important benefits to the aptamer approach include; in \textit{vitro} production which allows for greater target selection, uniformity of activity regardless of batch size, ability to bind ligands under a variety of conditions, enhanced shelf-life, and the ability to undergo chemical modifications which can enhance binding affinity and confer other diagnostic capabilities.\textsuperscript{14}

Table 4 - 1: Comparison between Aptamers and Antibodies for their use in small molecule binding. Reproduced from \textsuperscript{14}

<table>
<thead>
<tr>
<th>Aptamers</th>
<th>Antibodies</th>
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</thead>
<tbody>
<tr>
<td>Binding affinity nanomolar to picomolar range</td>
<td>Binding affinity nanomolar to picomolar range</td>
</tr>
<tr>
<td>Selection process in vitro therefore can target any protein</td>
<td>Selection require biological system difficult to target toxins and non-immunological targets (limited)</td>
</tr>
<tr>
<td>Can select for ligands under various conditions</td>
<td>Limited to physiological conditions for optimizing antibodies</td>
</tr>
<tr>
<td>SELEX process is cost and time effective</td>
<td>Screening monoclonal antibodies is time consuming and expensive</td>
</tr>
<tr>
<td>Uniform activity regardless of batch preparation</td>
<td>Activity can vary between batches</td>
</tr>
<tr>
<td>Pharmacokinetic parameters can be changed on demand</td>
<td>Difficult to modify pharmacokinetic properties</td>
</tr>
<tr>
<td>User defined targets</td>
<td>Immune system defined targets</td>
</tr>
<tr>
<td>Can undergo chemical modification</td>
<td>Limited to no modifications available</td>
</tr>
<tr>
<td>Return to form after temperature insult</td>
<td>Temperature sensitive, denaturation possible</td>
</tr>
<tr>
<td>Unlimited shelf-life</td>
<td>Limited shelf-life</td>
</tr>
<tr>
<td>Aptamer-specific antidote can be developed to reverse the interaction</td>
<td>No rational method to reverse interaction</td>
</tr>
</tbody>
</table>

With the expansion of aptamer target complexity,\textsuperscript{15} chemical modification to enhance aptamer properties is a new area of research. Modifications have included
attaching an amino or fluoro group to the 2'-position of sugars to confer nuclease degradation resistance\(^\text{16}\) and attaching a fluorophore to provide fluorescent capabilities.\(^\text{17}\) Providing aptamers with an easily detectable signal promotes their use as biosensors with industrial applications for detecting environmental contaminants, waste management, and drug screening.\(^\text{18}\) A novel approach for aptamer modification is formation of covalent linkages between an aptamer and its target.\(^\text{28}\) One advantage of the aptamer selection processes is the ability to screen an aptamer to a complex target with high binding affinity without knowing the precise identity of the binding molecule.\(^\text{19,20}\) Being able to obtain the target identity is important for the advancement of aptamers as analytical tools and biosensors for disease sensitive proteins. Furthermore, covalent attachment would enhance therapeutic capabilities of aptamers, used to bind and inhibit protein function, to a greater degree than simply enhancing non-covalent interactions. Intramers are intracellularly applied aptamers that specifically bind proteins, and disrupt intracellular signalling pathways.\(^\text{21,22}\) Intramers are used therapeutically to halt detrimental cellular processes and as research aids identifying specific proteins involved in signal cascades. Modified intramers have been developed to confer signalling properties, and their activity could be enhanced by covalent interactions.

An example of the potential for target identification using aptamers is Burkitt’s lymphoma. This disease is an extremely fast progressing cancer of the blood that requires early detection to maximize therapeutic potential. Burkitt’s lymphoma cells are immunophenotypically indistinguishable from many other large B-cell lymphomas and are diagnosed by inspecting the morphology of blood cells and the patient’s symptoms.\(^\text{23}\) An aptamer approach was successfully employed by Tang and co-workers to aid in early diagnosis of Burkitt’s lymphoma which demonstrated the usefulness and power of the aptamer-based approach.\(^\text{20}\) They used SELEX to produce aptamers that bound
specifically to proteins expressed by cancerous cells which, they postulated improved
diagnosis time and would increase survival rates.

This application has also led to research on identification of membrane proteins
that play an important role in disease progression of a healthy cell into a diseased cell.\textsuperscript{24-26} Molecular recognition towards specific cells is a key step for disease diagnosis and
therapy. Because cancer cells are genotypically abnormal, they express unique proteins
that distinguish them from normal, unaffected cells.\textsuperscript{27} Efforts in modern chemotherapy
have focused on finding more specific cures to limit side-effects observed in current
treatments. Distinguishing these cells based on altered cell surface proteins facilitates
this process.\textsuperscript{27} Proteomic analysis methods have shown success in this regard,
identifying large numbers of proteins involved in these processes and their expression
patterns. Identification of specific protein markers that correlate to diseased states
remains a challenge that has been approached by generating specific antibodies.\textsuperscript{28,29} As
stated previously, there are various challenges with regard to using antibodies, which
are overcome by using aptamers for the same purpose.\textsuperscript{30} Therefore, interest is growing
for the generation of aptamer libraries to these cell surface proteins and the use of
covalent linkages to tag and identify the protein targets.

Examples of aptamer covalent linkages to protein targets include work by
Mallikaratchy and co-workers who employed the use of a photoreactive iodouridine
molecule to induce cross-link formation between an aptamer and cell surface proteins in
cancer cells.\textsuperscript{31} The group also modified aptamers, allowing them to be bound to a solid
support which increased collection efficiency. It is important to note that this method of
covalently linking the aptamer protein complex was essential in overcoming stability
issues from conventional extraction procedures which used membrane-mimicking
detergents. However, the method suffered from placement limitations of the modification
within the aptamer complex as the bulky iodouridine group caused strand destabilization at certain sites.

Further work by Vinkenborg and colleagues addressed this limitation by employing a phenyl azide linker on the 5\' end of the aptamer that could form cross-links with the target protein upon activation by UV-light. The phenyl azide did not destabilize the secondary structural folding motif since the modification was located away from the interacting section of the aptamer (Figure 4-2). The group demonstrated the use of these modified aptamers in a variety of biological media, including cytoplasm, cell surfaces, and the blood stream, exemplifying the broad range of applications for this technology.
One well-studied aptamer is the thrombin binding aptamer (TBA).\textsuperscript{33} Thrombin is a key regulatory enzyme in the blood coagulation cascade and converts fibrinogen into fibrin.\textsuperscript{34,35} Thrombin inactivation can lead to several pathological states such as, hemorrhage or abnormal clot growth (thrombosis).\textsuperscript{36} As thrombosis and connected diseases are a major source of mortality in western countries, molecules that interact with this protein and modulate its activity represent a significant anticoagulant therapeutic strategy.\textsuperscript{36} A single stranded aptamer was developed in 1992 and was one of the first therapeutic aptamers developed.\textsuperscript{33} One biologically active aptamer for thrombin is a 15-mer oligonucleotide, comprised of only guanine and thymine bases, containing a sequence of 5’-GGTTG\textsubscript{8}TG\textsubscript{8}TGGTTGG-3’. The secondary structure has been determined by X-ray\textsuperscript{37} and NMR\textsuperscript{38} experiments and is well defined. The strand folds into an anti-parrallel G-quadruplex with two G-tetrads that contain alternating \textit{syn} and \textit{anti} G’s (Figure 4-3A).\textsuperscript{39} Also, this structure forms a complex with potassium ions that stabilizes correct folding.\textsuperscript{40} The two anti-parallel G-tetrads are connected by a single T-G-T loop (Figure 4-3B). The binding site for TBA on thrombin protein was found to be the anion-binding exosite I.\textsuperscript{41}
Crystallographic studies have also been performed on the aptamer-protein complex showing which nucleobases are in close proximity to the protein surface and how they interact with amino acids at the binding site. For example, isoleucine residues are involved with important hydrophobic contacts at $T_3$ or $T_7$ in human thrombin. These amino acids are both valine in bovine thrombin, and the lack of proper hydrophobic contact with bovine thrombin could explain observed differences in binding affinities between species. The depth of knowledge about the secondary structure of TBA and crystallographic data showing pertinent interactions between TBA and thrombin, allows chemical enhancement experiments to be carried out in an intelligent manner. As modifications have been shown to play an important role enhancing biological and
therapeutic properties for TBA,\(^{40}\) it was chosen as an excellent candidate to study specific effects upon nucleobase alteration.

Modification of TBA has been attempted previously by directly modifying a nucleobase\(^ {43}\) or modifying the sugar phosphate backbone.\(^ {44}\) These chemical alterations were originally developed to enhance folding stability and anti-thrombin activity.\(^ {43}\) The interaction between TBA and thrombin blocks thrombin activity.\(^ {38}\) Prolonging or strengthening this interaction has therapeutic benefits by lowering administered doses and increasing therapeutic life time.\(^ {38}\) The two G tetrad structures in TBA have four guanines that require syn conformation to form correct Hoogsteen binding in the secondary structure (Figure 4-3).\(^ {25}\) Previous work by the Manderville laboratory incorporated a 2-furyl modification at the C8-position of guanines located at G\(_5\), G\(_6\) and G\(_8\) in TBA (Figure 4-3C).\(^ {45}\) It was found that modification on a guanine favoring a syn conformation stabilized folding of the aptamer by lowering the energy requirement for the folding process.\(^ {45}\) The work in this study utilized the emissive fluorescent properties of furyl modification at C8-guanine to probe secondary structure folding and demonstrated beneficial signalling properties to detect duplex to quadruplex folding interactions.\(^ {45}\)

Previous work in this Thesis concerning secondary oxidative processes with phenol adducts (Chapter 3) demonstrated that attachment of an aryl moiety to the C8 position could lower the oxidation potential of the base, creating a susceptible site for further oxidative processes. Oxidation of the catechol adduct (3\(^ \prime\),4\(^ \prime\)-DHPH-dG) formed reactive quinones that were susceptible to nucleophilic attack. Additionally, successful incorporation of a furyl modification into TBA by the Manderville laboratory suggested that aryl modifications could be well tolerated by aptamers containing syn-specific guanines in their secondary structure.\(^ {45}\) Modifications using furans possessed some interesting properties, as furyl modified nucleosides examined by Sproviero and
colleagues contained beneficial fluorescent properties and were used as probes. Work by other groups demonstrated that furans are oxidised to form α,β-unsaturated carbonyls, reactive sites that are similar to oxidation products observed previously for catechol-modified nucleosides.46

Studies conducted by Madder and co-workers, incorporated a furyl modification onto the 2' position of the sugar backbone of an oligonucleotide by an acrylic linkage.46 The modification was oxidized chemically and photochemically to form site specific DNA cross-links.47

![Diagram](image_url)

**Figure 4 - 4: Mechanism of furan oxidation methodology for formation of DPCs.**48

More recent work, published by the same group, used similar modifications to generate DNA-protein cross-links (DPCs) site-specifically by exploiting the nucleophilic side chain functionality of amino acids (Figure 4-4).48 Furyl-modified nucleosides represent a latent functionality that can be activated on demand, to form a ring opened α,β-unsaturated dialdehyde species upon oxidation.47 Reactive aldehydes are present in cells from various exogenous sources like cigarette smoke and automobile exhaust and show cross-linking ability to DNA which is theorised to be a mechanism for their toxicity.49,50 Furthermore, α,β-unsaturated carbonyl formation is a popular cross-linking group and is responsible for mutagenicity observed by other carcinogens.51

Peterson and co-workers have also published a large body of work regarding the metabolic pathways of furan itself leading to observed carcinogenesis in rodent
models. These pathways were utilized by the Madder group to functionalize their oligonucleotides. CYP 450 activation of furan to the reactive cis-2-butene-1,4-dial molecule was shown to posses dual reactivity with dA and dG, forming bicyclic adducts in vivo by nucleophilic attack to the unsaturated carbonyl group. Studies performed by this group quantified the amount of metabolites formed through various methods of microsomal oxidation. Kinetic studies used glutathione (GSH) to trap the very reactive metabolic reaction intermediate before any further reactions could occur. The mechanism involved was a Michael addition reaction. Interestingly, this glutathione conjugate quickly reacted with the amine group of another equivalent of GSH to form bis GSH conjugates. These studies confirmed the oxidative reactivity of furan and susceptibility of the ring-opened group to nucleophilic attack. The same group performed further studies and observed that these glutathione bound furan conjugates could undergo a second round of nucleophilic attack, cross-linking with amino acids like lysine and glutamine, and forming a stable pyrrole conjugate. The conjugates were stable enough to serve as biomarkers for furan exposure in rat studies (Figure 4-5).

Figure 4 - 5: Ring opening of furan to form cis-butene-1,4-dial and subsequent conjugation with glutathione (GSH) and lysine to form double conjugated pyrrole adducts
The results from studies by Madder and Peterson on the oxidative reactivity of furan, as well as current synthetic successes in the Manderville laboratory incorporating aryl modifications into TBA, encouraged an idea. Furan could be easily incorporated into a nucleoside by established coupling reactions. The latent functionality of the newly synthesized furan nucleoside provides excellent control over activation conditions, and commencement of the cross-linking pathway to the reactive dialdehyde could be achieved chemically, enzymatically and photochemically. The natural reactivity of the ring-opened dialdehyde toward amino acid nucleophiles, with the demonstration of DPC formation in the literature, supported its use as a covalent linking tool.

It was decided to employ this well developed chemical reactivity by inserting the furan moiety onto a functional oligonucleotide (aptamer) and activating the furan by oxidation, to covalently link the modified aptamer to its protein target. As mentioned previously, functionalization of aptamers extends the biosensing applications of these molecules, and allows for isolation and characterization of unknown protein targets such as, those on the surface of cancer cells. This technology could, therefore, be used to aid in detection and characterization of unique cancer biomarkers, allowing early detection, and individualized therapeutic treatments.

Chapter 4 presents the design and synthesis of both the 2 and 3 furan isomeric adducts at the C8 position of dG. Preliminary oxidative studies on the nucleosides is shown, and demonstrates the greater utility of the 3-furyl modification compared to the 2-furyl modification for efficient oxidative activation. Furthermore, N-acetylcysteine (NAC) is used in conjunction with Nα-acetyl-L-lysine (NAL) to form the desired pyrrole dG conjugate (NAC-NAL-3fur-dG). Deglycosylation was observed as reaction progressed; however, the NAC-NAL-3fur-G conjugate was the only major species observed upon reaction completion. Although sugar loss to the conjugate was surprising, this species
was developed as the analytical standard for further reactions. Full characterization of the deglycosylated NAC-NAL-3fur-G conjugate was accomplished using tandem MS and 2D NMR experiments. Synthesis of the modified 3-furan phosphoramidite and incorporation into the TBA aptamer is also reported. Finally, oxidation reactions were carried out on the modified TBA strand. Trapping studies showed reactivity with NAC and NAL as observed with the nucleoside experiments; and, work with the thrombin protein provided suggestive evidence for site-specific cross-linking ability, to form a covalent linkage as hypothesized.
4.2. Experimental Procedures

4.2.1 General methods

A detailed description of the experimental procedures performed in Chapter 4 are given in Appendix A. Detailed explanations of specific methodology is given below.

4.2.2 Synthesis of 8-(3-furyl)-2'-deoxyguanosine (3fur-dG) Phosphoramidite and 8-(2-furyl)-2'-deoxyguanosine (2fur-dG).

To synthesize the two furan modified nucleosides, and the protected 3-furan phosphoramidite, established literature procedures were followed,\textsuperscript{56} detailed previously in sections 1.3. and 2.2. High resolution mass spectra for all samples were recorded at the Biological Mass Spectrometry Facility at the University of Guelph (Guelph, ON).

4.2.2.1 Suzuki-Miyaura coupling of 8-Br-dG with Furan Boronic Acids.

These reactions were carried out according to the literature, and are briefly described here.\textsuperscript{57} The initial reaction employed 0.07 equiv of 3,3',3''-phosphanetriyltris(benzenesulfonic acid) trisodium salt (TPPTS), 0.03 equiv of palladium(II)acetate (Pd(OAc)\textsubscript{2}), 2 equiv of Na\textsubscript{2}CO\textsubscript{3} and 3:1 equiv furan boronic acid: 8-bromodeoxyguanosine (8-Br-dG). The reagents were added simultaneously to a reaction flask containing a freshly degassed 2:1 water:acetonitrile solution. The reaction vessel was purged with argon, and stirred under inert atmosphere for 4 h at 80 °C. The pH was adjusted to 7.2 and the resulting suspension was placed on ice overnight. The pure product was filtered and collected as a solid. After drying the product, no further purification was necessary based on the NMR spectra, shown in Appendix C.
8-(2-furyl)-2'-deoxyguanosine (1). Starting from 8-Br-dG (1.0 g, 3.0 mmol), TPPTS (0.12 g, 0.067 equiv, 0.208 mmol), Pd(OAc)$_2$ (0.02 g, 0.026 equiv, 0.078 mmol), Na$_2$CO$_3$ (0.64 g, 2 equiv, 6.06 mmol) and 2-furyl boronic acid (1.00 g, 3 equiv, 0.078 mmol) afforded the pure product as an off white solid (0.84 g, 84%); $^1$H NMR (600MHz, DMSO-d$_6$) $\delta$ 10.77 (s, 1H), 7.89 (s, 1H), 7.82 (s, 1H), 6.94 (m, 1H), 6.67 (m, 1H), 6.43 (s, 2H), 6.35 (t, J = 7.0 Hz, 1H), 5.18 (d, J = 4.6 Hz, 1H), 4.93 (t, J = 5.6 Hz, 1H), 4.37 (m, 1H), 3.78 (m, 1H), 3.61 (m, 1H), 3.48 (m, 1H), 3.15 (m, 1H), 2.07 (m, 1H); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 156.4, 153.2, 151.7, 144.4, 144.1, 137.8, 117.4, 112.1, 111.8, 87.8, 84.4, 71.2, 62.1, 36.9; HRMS (ESI) calc for C$_{14}$H$_{15}$N$_5$O$_5$ 334.1143 found 334.1140 (MH$^+$).

8-(3-furyl)-2'-deoxyguanosine (2). Starting from 8-Br-dG (1.04 g, 3.0 mmol), TPPTS (0.1184 g, 0.0667 equiv, 0.208 mmol), Pd(OAc)$_2$ (0.0176 g, 0.0260 equiv, 0.0784 mmol), Na$_2$CO$_3$ (0.640 g, 2 equiv, 6.06 mmol) and 3-furyl boronic acid (1.01 g, 3 equiv, 9.02 mmol) afforded the pure product as a grey solid (0.81 g, 82%); $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 10.70 (s, 1H), 8.13 (s, 1H), 7.82 (s, 1H), 6.83 (s, 1H), 6.39 (s, 2H), 6.14 (t, J = 7.2 Hz, 1H), 5.17 (d, J = 4.6 Hz, 1H), 4.96 (t, J = 5.8 Hz, 1H), 4.36 (m, 1H), 3.78 (m, 1H), 3.61 (m, 1H), 3.55 (m, 1H), 3.18 (m, 1H), 2.06 (m, 1H); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 157.0, 153.7, 152.2, 143.1, 144.5, 141.1, 117.4, 117.1, 111.5, 88.1, 84.5, 71.0, 62.2, 36.9; HRMS (ESI) calc for C$_{14}$H$_{15}$N$_5$O$_5$ 334.1143 found 334.1153 (MH$^+$).

4.2.2.2 DMF protection of 3-furyl modified nucleoside

$N^2$-(Dimethylforamidyl) protection of the exocyclic amine on 3fur-dG (2) followed established literature procedures analogously to section 2.2. and will not be reiterated here.58
**N^\text{2}-(Dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine (2a).** Starting from 1.67 g (5.01 mmol) of (2) and 3.43 mL (4 equiv, 20 mmol) of \(N,N\)-dimethylformamidyl diethyl acetal afforded the pure product as an off white solid (1.89 g, 97%); \(^1\)H NMR (400 MHz, DMSO-\text{d}_6) \(\delta\) 11.45 (s, 1H), 8.50 (s, 1H), 8.17 (s, 1H), 7.86 (s, 1H), 6.87 (s, 1H), 6.22 (t, \(J = 7.4\) Hz, 1H), 5.28 (m, 1H), 4.87 (t, \(J = 5.8\) Hz, 1H), 4.46 (m, 1H), 3.81 (m, 1H), 3.63 (m, 1H), 3.54 (m, 1H), 3.20 (m, 1H), 3.16 (s, 3H), 3.05 (s, 3H), 2.12 (m, 1H); \(^{13}\)C NMR (100 MHz, DMSO-\text{d}_6) \(\delta\) 158.0, 157.3, 156.7, 150.5, 144.1, 142.6, 141.3, 120.0, 116.2, 110.8, 87.5, 84.2, 70.7, 61.7, 40.8, 37.0, 34.6; HRMS (ESI) calc for \(C_{17}H_{20}N_6O_5\) 389.1565 found 389.1577 (MH\(^+\)).

**4.2.2.3 DMT protection of DMF-protected 3-furyl nucleosides**

Dimethoxytrityl protection strategy was again employed on \(N^\text{2}-(\text{dimethylformamidyl})-8-(\text{3-furyl})-2'-\text{deoxyguanosine}\) according to established literature procedures,\(^59\) with slight modifications previously detailed in section 2.2.

**5'-O-(4,4'-Dimethoxytrityl)-N^\text{2}-(\text{dimethylformamidyl})-8-(3-furyl)-2'-deoxyguanosine (2b).** Compound 2a (0.934 g, 2.41 mmol) and 0.987 g (1.2 equiv, 2.91 mmol) of DMT-Cl were used to afford the pure product as a white solid (1.22 g, 73%); \(^1\)H NMR (600 MHz, Acetone-\text{d}_6) \(\delta\) 10.08 (s, 1H), 8.52 (s, 1H), 8.19 (s, 1H), 7.72 (s, 1H), 7.37 (m, 2H), 7.24-7.11 (m, 7H), 7.73 (m, 4H), 6.35 (m, 1H), 5.07 (m, 1H), 4.12 (m, 1H), 3.75 (s, 6H), 3.71 (m, 1H), 3.34 (m, 1H), 3.27 (m, 1H), 3.09 (s, 6H), 2.47 (m, 1H), 2.38 (m, 1H); \(^{13}\)C NMR (151 MHz, Acetone-\text{d}_6) \(\delta\) 159.5, 159.4, 158.7, 151.4, 146.3, 144.5, 143.3, 143.2, 137.0, 136.9, 130.9, 130.7, 129.0, 128.4, 127.4, 121.8, 118.3, 113.7, 113.6, 111.9, 87.1, 86.5, 85.4, 64.9, 55.5, 47.1, 41.4, 35.1; HRMS (ESI) calc for \(C_{38}H_{38}N_6O_7\) 691.2893 found 691.2889 (MH\(^+\)).
4.2.2.4 Phosphitylation of DMF-DMT protected 3-furyl modified nucleosides

Phosphitylation reactions were carried out on 5'-O-(4,4'-Dimethoxytrityl)-N2-(dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine as described in the literature,\textsuperscript{60-62} with modifications detailed previously in section 2.2.

3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino] -5'-O-(4,4'-dimethoxytrityl)-N2-(dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine (2c). Compound 2b (0.5 g, 0.741 mmol), 0.404 mL (4 equiv, 2.96 mmol) of TEA and 0.243 mL (1.5 equiv, 1.09 mmol) of 2-cyanoethyl-N,N-diisopropyl-chloro-phosphoramidite were used to afford the pure product as a white foamy solid (0.426 g, 68%); \textsuperscript{1}H NMR (600 MHz, Acetone-d\textsubscript{6}) \( \delta \)
10.13 (s, 1H), 8.57 (m, 1H), 8.18 (m, 1H), 7.72 (s, 1H), 7.35 (m, 2H), 7.24-7.13 (mm, 8H), 6.75-6.68 (m, 4H), 6.35 (t, J = 7.2 Hz, 1H), 5.34 (m, 1H), 4.17 (m, 1H), 3.84 (m, 1H), 3.73 (m, 7H), 3.60 (m, 3H), 3.29 (m, 2H), 3.17 (s, 3H), 3.09 (s, 3H), 2.51 (m, 2H), 1.17 (m, 9H), 1.04 (m, 4H); \textsuperscript{31}P NMR (121MHz, Acetone-d\textsubscript{6}) \( \delta \) 149.31, 148.92; HRMS (ESI) calc for C\textsubscript{47}H\textsubscript{57}N\textsubscript{8}O\textsubscript{7}P 877.4095 found 877.4090 (MH\textsuperscript{+}).

4.2.3. Synthesis and purification of 3-furyl modified Thrombin Binding Aptamer (mTBA).

Modified 15mer TBA oligonucleotides (mTBA), were synthesized using the solid phase method performed on a 1\textmu mol scale on a BioAutomation Corp. MerMade 12 automatic synthesizer, and employing the 3-furyl-modified phosphoramidite. Recall that the TBA sequence is 15 bases long with a sequence of 5'-GGTTG\textsubscript{5}G\textsubscript{6}TG\textsubscript{8}TGGTTGG-3'. The 3-furyl modified dG base was inserted individually into positions 5, 6 and 8. Standard coupling times were used for synthesis, and the machine was operated by Michael Sproviero in the Manderville laboratory, at the University of Guelph. Following synthesis, mTBAs were cleaved from the solid support and deprotected using 2 mL of a
30% ammonium hydroxide solution at 55 °C for 12 h. Following filtration with a 0.2 µm PVDF microfilter from Mandel Scientific, the crude sequence mixture was purified by RP-HPLC as described in section 1.3.3.

4.2.4. Photophysical Analysis and Characterization of Conjugates Produced by Oxidation Reactions.

4.2.4.1 UV-visible Absorbance Measurements of Modified Nucleosides and Conjugate Molecules

For photophysical analysis, stock solutions of 3fur-dG, 2fur-dG, NAC-3fur-dG, NAC-NAL-3fur-dG and NAC-NAL-3fur-G were prepared to a 5 mM concentration in DMSO. Samples for analysis were prepared by adding 20 µL of stock solution to 1980 µL of 0.05 M Na₂PO₄ buffer containing 0.1 M NaCl at pH 7. The final concentration of the modified nucleoside in solution was 50 µM. Samples for analysis were prepared by adding 20 µL of stock solution to 1980 µL of 0.05 M Na₂PO₄ buffer containing 0.1 M NaCl at pH 7. The final concentration of the modified nucleoside in solution was 50 µM.

4.2.4.2 Fluorescence Measurements of Modified Nucleosides and Conjugate Molecules

Stock solutions of 3fur-dG, 2fur-dG, NAC-3fur-dG and NAC-NAL-3fur-G were prepared in DMSO to a 2.5 mM concentration. The final concentration of the modified nucleoside in the stock solution was 50 µM. Samples for analysis were prepared by adding 20 µL of this stock solution to 1980 µL of 0.05 M Na₂PO₄ buffer, containing 0.1 M NaCl at pH 7 to afford a final concentration of 10 µM.
4.2.5. HPLC Analysis for Nucleoside Experiments

Reaction monitoring, adduct purification and conjugate isolation experiments for the 2fur-dG and 3fur-dG were accomplished using reverse phase HPLC analysis. The compounds were separated on a C18 column using a mobile phase of 95% triethylammonium acetate (TEAA): 5% acetonitrile (ACN), with an increasing gradient of 30% triethylammonium acetate: 70% acetonitrile for elution. Peaks were detected by monitoring UV absorbance at $\lambda_{\text{max}}$ 258 nm, $\lambda_{\text{max}}$ 290 nm and fluorescence $\lambda_{\text{ex}}$ at 320 nm, $\lambda_{\text{em}}$ at 360 nm.

4.2.6. 2D NMR Experiments for Full Characterization of NAC-NAL-3fur-G Conjugate

Samples for $^1$H, $^{13}$C, HMBC and HMQC analysis were prepared to a concentration of 60 μM in DMSO, 200 μL total volume. The solution was loaded into a shigemi tube. $^1$H and $^{13}$C NMR experiments were run on a Bruker Avance DPX 600 MHz NMR machine, and deuterated solvent signal (DMSO-d$_6$) was used as the primary reference. 2D NMR experiments (COSY, HMQC and HMBC) were performed according to standard pulse programs with Bruker software. $^1$H NMR and COSY spectra were acquired using water suppression techniques. Experiments were performed in the NMR facility, at the University of Guelph, with assistance from Valerie Robertson.

4.2.7. Tandem MS/MS Analysis of Trapped Conjugates

Samples of NAC-3fur-dG and NAC-NAL-3fur-G were prepared in DMSO to a final concentration of 60 μM. Aliquots (10 μL) were diluted with 990 μL of MS ionization buffer, composed of 95% MilliQ filtered water, 5% MeOH and 1% ammonium acetate to a final concentration of 0.6 μM. A Bruker AmaZon quadrupole ion trap SL spectrometer was used for the analysis. Masses were acquired in negative ionization mode with an electrospray ionization source (Bruker Daltronics, Milton, Canada). Ionization analysis
was carried out using the following settings on the ESI: nebulizer gas flow 40 psi, drying
gas flow 10 L/min, dry gas temperature 200 °C, spray voltage was -4000 V. Mass range
was 70-2000 m/z and scan rate was 8100 m/z/s. Injection rate was 10 µL/min. The MS^n
spectra were obtained by CID with helium gas after isolation of the appropriate precursor
ions. Collision energies varied from 0.20 V to 0.60 V depending on the species being
fragmented. Direct injection was used to infuse the sample into the ESI source.

4.2.8. Polyacrylamide Gel Electrophoresis of 3fur-TBA Conjugates with Thrombin
Protein

Samples for PAGE analysis were prepared by mixing an aliquot of TBA oxidation
reaction and protein standard solution with an equal volume of LaemmLi sample buffer
[0.125 M Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 25 mM EDTA, 2% β-
mercaptoethanol]. This solution was immersed in a hot water bath at 100 °C for 4 min.
Electrophoresis of TBA oxidation reaction samples and protein standards was conducted
using a 10% Mini-PROTEAN TGX Precast Gel (Bio-Rad) in a Tris-glycine running buffer.
Bands corresponding to protein were identified by post-electrophoresis dying with
Coomassie Brilliant Blue R-250. All PAGE analysis was carried out by Lisa Bertolo at the
University of Guelph.
4.3. Results and Discussion

4.3.1 Synthesis and Characterization of Furan Modified Nucleosides and Furan Modified TBA

(a) C8-(2-furyl)-2'-deoxyguanosine (1) and C8-(3-furyl)-2'-deoxyguanosine Adducts (2)

The two modified nucleosides were synthesized according to previously published literature procedures using a palladium catalyzed Suzuki-Miyaura coupling procedure. 8-Br-dG was employed as the starting material, and the appropriate 3-furyl boronic acid was attached as shown in Scheme 4-1. Precipitation of the final product resulted in excellent yield (> 80%), and purity, for the two adducts (NMR spectra, Appendix C). Although both adducts have been synthesized previously by Rivera and co-workers, irradiation was used and yields were poor. The method utilized for this project produced no problems during synthesis.

Scheme 4 - 1: Synthesis of 3fur-dG and 2fur-dG from 8-Br-dG.
(b) Synthesis of 3-furyl modified Phosphoramidite.

Synthesis of the 3fur-dG phosphoramidite followed standard methods shown in the literature. Extensive discussion of the synthetic steps for phosphoramidites was addressed in Chapters 1 and 2. Protection of the exocyclic amine using DMF and the 5′-OH group using DMTCl was followed by reaction with the phosphitylating agent at the 3′-OH. The complete synthetic scheme for 3′-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5′-O-(4,4′-Dimethoxytrityl)-N2-(Dimethylformamidyl)-8-(3-furyl)-2′-deoxyguanosine is shown in Scheme 4-2.

Scheme 4 - 2: Synthesis of 3fur-dG phosphoramidite (2c)

c) Synthesis of Modified TBA Oligonucleotides

3-furyl modified-TBA nucleotides were synthesized according to established methods by Michael Sproviero in the Manderville laboratory. Strands were isolated in good yields after ammonium hydroxide deprotection and confirmation of correct structure was obtained by MS (Appendix B).
4.3.2 Nucleoside Oxidation and Trapping Studies

Oxidation studies of 2fur-dG (1) and 3fur-dG (2) were performed chemically using N-bromosuccinimide (NBS). The anticipated reaction pathway for the 3fur-dG derivative (Scheme 4-3) was expected to produce the fully conjugated species (5).

Scheme 4 - 3: Oxidation and trapping studies of (2) using NAC and NAL to form NAC-NAL-3fur-dG (5). Deglycosylation affords the final adduct standard (6).

![Scheme 4-3](image_url)

Stock solutions of (1) and (2) were prepared in DMSO, due to sparing solubility in aqueous solution for most C8-aryl modified nucleosides. Reaction solutions of (1) and (2) were prepared by diluting the stock to a concentration of 0.4 mM in Na$_2$PO$_4$ buffer at pH 7. A stock solution of NBS was prepared to the same concentration in Na$_2$PO$_4$ buffer, and 1 equiv was added. Subsequent additions of NBS took place every 10 min, while monitoring reaction progress by HPLC and UV-vis, until consumption of the starting material had occurred. Shown in Figure 4-7 is a time-course UV-vis trace that follows oxidation as NBS is added to (1) and (2) respectively.
Figure 4-6: UV-vis absorbance spectra as NBS is added to A) 2fur-dG (1) and B) 3fur-dG (2). Arrows show changes in absorbance upon reaction progress.

For both adducts, it was observed that addition of NBS caused the initial solitary peak to decrease in intensity, with formation of new absorbances. For (1), the original absorbance peak $\lambda_{\text{max}} = 293$ nm disappeared, and three new absorbance maxima were observed at $\lambda_{\text{max}} = 277, 310$ and $363$ nm (Figure 4-6A). For (2), the original absorbance at $\lambda_{\text{max}} = 274$ nm decreased, and a new maximum was observed, with $\lambda_{\text{max}} = 280$ nm and a prominent shoulder at 310 nm (Figure 4-6B). Cessation of spectral changes was interpreted as reaction completion, and NBS additions were stopped. Aliquots of the reaction mixtures were collected before NBS addition and upon reaction completion, according to UV-vis scans. These samples were then analyzed by HPLC. Adduct (1) required 5 equiv of NBS to reach a minimum absorbance value, while adduct (2) required only 4 equiv of NBS before no further spectral changes were observed. The NBS solution used for both experiments was the same, suggesting that 3fur-dG was more easily oxidized than 2fur-dG.
The oxidation reaction was also monitored by HPLC to determine the identity and spectral characterization of the oxidation products. Figure 4-7A and 4-7B show HPLC data for the oxidation of 2fur-dG (1). It was observed that two major products resulted from the oxidation reaction. One species eluted at 17.6 min (Figure 4-8B peak i), and the second oxidation product eluted at 26.5 min (Figure 4-8B peak ii). These two species were isolated and characterized by ESI-MS. Peak i) produced an ESI⁺-MS spectrum showing a parent ion of \( m/z = 350 \), corresponding to the desired ring-opened 1,4-dicarbonyl (Figure 4-7C). This molecule fragmented in the gas phase to afford \( m/z = 234 \), indicative of sugar loss (-116), which is characteristic for nucleosides.

![Figure 4-7](image)

**Figure 4-7:** A) HPLC trace showing (1) pre-oxidation with NBS. B) HPLC trace of (1) after addition of 5 equiv of NBS over 40 min. C) ESI⁺-MS for product peak i) from oxidation of (1). D) ESI⁺-MS for product peak ii from oxidation of (1).
Peak ii) in Figure 4-7B isolated from oxidation of (1) was also analyzed by ESI’-MS, and produced a parent mass ion $m/z = 430$, which was hypothesized to be brominated furan and not the desired ring-opened product. One major fragment, corresponded to $m/z = 314$, was observed upon MS$^2$ analysis and represented sugar loss (-116). Although this second oxidation product was not fully characterized, the mass observed ($m/z = 430$), combined with the isotopic pattern showing a second peak at (M+2) $m/z = 432$, confirmed the presence of the bromine atom. Sugar loss ($m/z = 314$), was suggestive of a nucleoside, and both results strongly suggest that peak (ii) was the brominated furan.

![Figure 4 - 8: A) UV-vis absorbance spectra of oxidation product peak (i) from (1). B) UV-vis absorbance spectrum of oxidation product peak (ii) from (1).](image)

The UV-vis spectrum for peak (i) (Figure 4-8A), produced two major peaks at $\lambda_{\text{max}}$ 248 nm and $\lambda_{\text{max}}$ 368 nm. Peak (ii) produced a $\lambda_{\text{max}}$ of 310 nm (Figure 4-8B). All of these maxima were also observed in the UV-vis spectrum monitoring oxidation of (1) (Figure 4-6A), and the spectrum observed after complete oxidation represents production of two different products that were resolved by HPLC. Oxidation of (1) demonstrated that bromination was able to compete effectively with ring opening which was not desirable.
Efficient, selective oxidation producing a single species, was the goal for this step of the conjugation pathway to simplify characterization of subsequent conjugation reactions.

Oxidation of (2) however, gave a much improved and efficient result. As shown in Figure 4-9B, only one major oxidation product was observed (i) which eluted at 17.3 min by HPLC. The ESI⁺-MS spectra for this peak showed parent ion at $m/z = 350$ with characteristic sugar loss to afford the fragment $m/z = 234$ (Figure 4-9C). Peak (i) was attributed to the desired ring-opened product. This result also demonstrated that 3fur-dG was oxidized more efficiently than 2fur-dG since fewer equivalents of NBS were required for complete oxidation. Furthermore, the reaction was more specific, as bromination was not observed to compete effectively with ring-opening for (2), and only the 1,4-dialdehyde was produced (Figure 4-9).

![Figure 4-9:](image)

**Figure 4 - 9:** A) HPLC trace of (2) before oxidation. B) HPLC trace of (2) after addition of 4 equiv NBS over 30min. C) ESI⁺-MS for oxidation peak i). D) UV-vis absorbance spectrum of oxidation peak i).
UV-vis characterization of the 1,4-dialdehyde oxidation product is displayed in Figure 4-9D and produced a $\lambda_{\text{max}} = 286$ nm with a prominent shoulder at 306 nm. Again, the absorbance profile of this species was observed in UV-vis spectra monitoring oxidation reaction progress (Figure 4-6B).

The large red-shift in absorbance for the 1,4-dicarbonyl, resulting from oxidation of (1) and (2), is attributed to donor acceptor (D-A) character in the conjugated system of the oxidized base. The electron-rich guanine base is the donor, and the carbonyl group in close proximity is the acceptor. Furthermore, the positioning of the acceptor at C8 increases the magnitude and aligns the direction of the transition dipole moment, which lowers the LUMO energy. The decrease in HOMO-LUMO gap lowers the excited energy state resulting in a longer absorption wavelength (368 nm from 1 and 306 nm from 2 Figure 4-7). After oxidation of (2), the electron acceptor carbonyl group is positioned farther from the donor guanine and exerts a significantly lower electron-withdrawing effect which produced a smaller bathochromic shift. This effect for both ring-opened oxidation products was diagnostically very useful and had significant advantages in further DNA studies. Oxidation reaction progress could be monitored by scanning for a unique wavelength separated from DNA (260 nm) which would be present in much higher abundance since only one base was modified.

Although (2) gave only the ring-opened oxidation product, it was desirable to explain why (1) produced a competitive oxidation pathway. It was postulated that steric effects from the dG moiety could play a role, as the position of guanine was the only difference between both isomeric species. Scheme 4-4 shows proposed mechanisms for the two competing oxidative processes observed, adapted from a mechanism proposed by Kelly et al. It is known that bromination of furan with NBS will yield the halogenated furan in organic solvents. In aqueous media, a second pathway competes with bromination to afford the desired 1,4-dialdehyde. The mechanism, shown in Scheme 4-
4, illustrates two possible reaction pathways for oxidative attack by NBS. Bromination of the furan ring, forms the oxonium ion that undergoes addition of water at the 2 position, followed by loss of HBr and oxidative ring fission, to afford the 1,4-dialdehyde intermediate (Scheme 4-4, Path A).\textsuperscript{71}

**Scheme 4 - 4: Proposed oxidation pathways taken by (1) and (2) upon addition of NBS.** 2fur-dG undergoes competition between path A and path B due to sterics while 3fur-dG proceeds through a single oxidative pathway to afford the 1,4-dicarbonyl-dG.

We propose that if the 2 position of the furan is sterically hindered by the R group (dG) attached at the site of nucleophilic attack, a competing pathway, driven by re-aromatization of the furan ring and elimination of a proton, occurs (Scheme 4-4, path B). This steric effect has been observed previously by Kobayashi and co-workers while using NBS to oxidize substituted furans during the synthesis of higher ordered pharmaceuticals.\textsuperscript{72} Re-aromatization competes with nucleophilic attack from H\textsubscript{2}O to afford the brominated furan in aqueous solution. Lack of steric hindrance in 3fur-dG (2)
produces a more accessible site for H$_2$O attack at the double bond on the oxonium ion, and only the 1,4-dialdehyde (3) is observed.

**Figure 4 - 10: HPLC traces illustrating** A) (2) after addition of 4 equiv NBS over 40 min. (B) 40 equiv of NAC added to reaction mixture with stirring for 2 h at 37 °C. (C) 20 equiv NAL added to reaction mixture and stirring overnight at 37 °C. D) Reaction mixture following 10% formic acid addition for 1 hr at 70 °C.

Both (1) and (2) gave the desired 1,4-carbonyl oxidation product, however the inefficiency of the reaction with (1) promoted the use of (2) only for subsequent conjugate experiments. Trapping studies were performed with NAC by adding 40 equiv of NAC to the reaction vessel upon completion of oxidation by UV-vis analysis. The reaction was left to stir for 2 h at 37 °C and was monitored by UV-vis and HPLC. Upon completion of 2 h reaction time, samples were injected into the HPLC to monitor reaction progress. Two new peaks were observed eluting at 16.2 min and 17.4 min (bi, bii, Figure 4-10). In addition, disappearance of the 1,4-dialdehyde peak at 17.3 min was observed suggesting that product peaks arose from reaction between NAC and 1,4-dialdehyde (Figure 4-10B). The new peaks (bi and bii) were collected and analyzed by MS. The
ESI²-MS² spectrum was identical for both peaks and is shown in Figure 4-11A. The parent \([\text{M-H}^+]\) ion at \(m/z = 511\) fragmented to produce \(m/z = 395\) and \(m/z = 266\), which corresponded to sugar loss (−116), and breakage of the NAC component (−129, Figure 4-11A). Loss of 129 is characteristic for NAC conjugates which aided in positive identification of (4) from this spectrum.\(^{73}\) Absorbance characteristics for both peaks were identical, and are shown in Figure 4-11B. A slightly blue-shifted \(\lambda_{\text{max}} = 268\) nm, and a prominent shoulder at 278 nm were observed.

![Figure 4 - 11: A) ESI—MS for peak bi) in Figure 11B representing product (4) which is the NAC trapped adduct of ring opened species from oxidation of (2) with NBS. B) UV-vis absorbance spectrum of peak bi).](image)

The attachment of NAC to the 1,4-dialdehyde causes a significant blue shift in the absorbance spectra due to decreased conjugation from double bond breakage upon nucleophilic attack. Decreased conjugation of a molecule has been shown to induce a blue shift from increased \(\pi-\pi^*\) transitional energy and a shorter, higher energy wavelength.\(^{74}\) This hypsochromic shift is not surprising given the proposed structure of the NAC conjugate (4), shown also in Figure 4-11. Recall that two peaks with identical MS and photophysical characteristics were observed upon trapping with NAC (Figure 4-10). It was hypothesized that attack to both positions across the double bond of the
unsaturated dicarbonyl compound gave rise to two NAC conjugates observed in the HPLC trace (Scheme 4-5). Further analysis was not conducted to elucidate the identity of both products, as focus remained on the reactivity of conjugate (4).

Figure 4-12: ESI−MS for isolated peak c) from the addition of 20 equiv NAL to the reaction mixture after stirring for 2 h at 37 °C.

Scheme 4-5: Two different site of nucleophilic attack by NAC on (3) producing two NAC trapped conjugates of the same mass.

Following confirmation of the successful NAC conjugate, addition of 20 equiv of \( N_\alpha\)-acetyl-L-lysine (NAL) was achieved by creating a stock solution in \( \text{Na}_2\text{PO}_4 \) buffer and diluting accordingly. The reaction was stirred for 2 h at 37 °C, and was monitored by
HPLC. Two new peaks were observed at 19.8 min and 22.3 min (Figure 4-10C). Collection of the peak at 22.3 min and MS analysis revealed that attachment of NAL to the NAC conjugate had occurred to form an NAC-NAL conjugate with 3fur-dG. MS analysis of this species in the negative mode afforded a parent ion that corresponded to the hypothetical structure shown in Figure 4-13. Furthermore, fragmentation of the parent ion demonstrated characteristic loss of sugar (−116) and NAC (−129) components to afford fragment peaks at $m/z = 547$ and $m/z = 418$ which strongly suggested the correct structure. Observation of the desired reaction sequence was very encouraging to proceed with further study in aptamer strands. However, a second peak was observed at 19.8 min which increased in intensity after allowing the reaction to stir overnight at 37 °C in slightly acidic conditions, due to the abundance of NAL in solution (Figure 4-10C-D). NAL has a $pK_a$ of 3.86 which, in the concentrations used, acidified the pH of the solution slightly despite the presence of buffer. Initially, it was thought that this peak corresponded to a second NAL isomer. However, stirring the reaction mixture increased the amount of the peak at 19.8 min, and the desired conjugate peak at 22.3 min disappeared. As shown in Figure 4-10D, reinjection after overnight stirring produced only one major species which eluted at 19.8 min.

Following overnight stirring, the peak at 19.8 min was collected, and MS analysis demonstrated that this species corresponded to the deglycosylated NAL-NAC-3-fur-dG adduct (6) (Figure 4-13). UV-vis and fluorescence analysis proved useful, as the absorbance spectra gave two absorbance maxima at $\lambda_{\text{max}} = 260$ nm and $\lambda_{\text{max}} = 310$ nm. Exciting the molecule at $\lambda_{\text{ex}} = 310$ nm produced fluorescence at $\lambda_{\text{em}} = 370$ nm (Figure 4-14). This result was significant for two reasons. First, fluorescence from the conjugated molecule now conferred a new diagnostic capability for facile detection of this species in complex media, and second, subsequent DNA reactions could employ fluorescence detection to confirm conjugate identity for new HPLC peaks.
The presence of (6) reiterated the success of the proposed reaction pathway and gave rise to a new idea. Acid lability could be used beneficially, as DNA bases are susceptible to acid hydrolysis, which would allow facile removal of successful conjugates produced in DNA through weak acid hydrolysis. As it was a major goal for these reactions to be performed in DNA, facile deglycosylation simplified DNA analysis experiments greatly. Conjugate (6) was therefore determined to be an important analytical standard for further experiments. This standard could be used to quantify conjugates produced from reaction at the various sites within the TBA aptamer.

Figure 4 - 13: A) ESI−MS for peak d) corresponding to deglycosylated (5). B) ESI−MS² for peak d). C) ESI−MS³ for peak d)
Unequivocal structural confirmation of (6) was ascertained by full MS$^n$ and NMR studies shown in Figure 4-14 and Table 4-2. ESI$^-$-MS$^n$ spectra produced characteristic losses of the NAC conjugate (-129) and a peak at m/z = 247, representative of fragmentation from the NAL component.$^{75}$ ESI$^-$-MS$^3$ fragmentation of the m/z = 418 peak showed loss of 42 from the NAL component to afford a major peak at m/z = 376. Once again, a peak m/z = 247 is observed which was attributed to cleavage of C2'' and C3''' on NAL (Figure 4-13). Loss of 42 had been previously observed for NAL as a fragment from cleavage of the N-acetyl group.$^{76}$ ESI$^-$-MS$^4$ studies on m/z = 376 produced losses of ammonia (-17) and isocyanic acid (-43) from the six-membered ring on guanine.$^{77}$ The MS studies confirmed the presence of both the NAC and NAL components attached to (3). Configuration of the conjugate was accomplished with one and two dimensional NMR experimental spectra including $^1$H, COSY, HSQC, and HMBC which are shown in Appendix C. Table 4-2 presents proton and carbon NMR assignments from 2D experiments, and the proposed structure is shown in Figure 4-14. These experiments confirmed attachment positions of NAC and NAL and verified formation of the pyrrole ring. The structure of (6) is in accordance with the desired attachment locations for NAC and NAL based on previous studies by Peterson.$^{55}$
Table 4 - 2: Chemical shifts calculated for (6)

<table>
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<th>$^{13}$C</th>
</tr>
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</tr>
<tr>
<td>Guanine</td>
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Figure 4 - 14: UV-vis and Fluorescence characteristics and structural assignments for adduct (6)
Upon full characterization of the deglycosylated adduct, a five point calibration curve was constructed to quantify products from subsequent reactions directly upon HPLC analysis. The calibration curve is shown in Figure 4-15 and gave an excellent $R^2$ value (0.998). The plot shows concentration versus the integrated area under the adduct peak.

An important aspect of the conjugation reaction not shown is the addition sequence. Although the reaction was analyzed in a step-wise fashion, with observations occurring after sequential additions of NAC and NAL, it was discovered that addition of appropriate equivalents of NAC and NAL simultaneously upon completion of oxidation, also gave the desired final product (6) in comparable yield. It was determined that this addition sequence would facilitate DNA experiments and all further reactions were carried out according to this method.

![Calibration curve derived from HPLC integration of (6).](image)

$y = 29.97x + 0.6782$

$R^2 = 0.998$

Figure 4 - 15: Calibration curve derived from HPLC integration of (6).
Successful reaction with chemical oxidants initiated questions regarding the use of other oxidant systems to generate analogous reactivity with (2). To test this, rose bengal (RB), a known singlet oxygen generator, was used. A stock solution of RB in Na$_2$PO$_4$ buffer was prepared to a concentration of 0.4 mM, and 1 equiv was added to the reaction mixture. The reaction was stirred in a photolysis chamber while photolysing the sample with light at 525 nm. The reaction was tested at different time intervals by HPLC and, after 45 min of irradiation, most of the starting material had been converted to the 1,4-dialdehyde (peak 17.4 min, Figure 4-16). Simultaneous addition of NAC and NAL were carried out upon oxidation completion. Aliquots for HPLC analysis were taken 2 h and 14 h after addition of NAC and NAL.

Figure 4-16 shows the oxidation reaction progress and trapping experiments. Although the addition of NAC and NAL produced a complex chromatogram (Figure 4-16C), stirring the reaction overnight at 37 °C gave the deglycosylated conjugate (6) in significant yield as the major product (Figure 4-16D, marked with *). This result demonstrated that photo-induced singlet oxygen favored a similar oxidation mechanism as chemical processes and suggested additional applications in biological media for adduct reactivity. Singlet oxygen generators are found endogenously in many biological systems and are used extensively in photodynamic therapy (PDT). These molecules could be used to oxidize furyl-modified DNA in cells, which would eliminate the use of chemical oxidants like NBS, that are incompatible with living systems.
4.3.3 Oxidation and Trapping Studies of 3furan-modified TBA Aptamers

The nucleoside studies showed that NBS and RB oxidation was efficient and selective for (2). Trapping with a sulphur nucleophile (NAC) proceeded according to the Michael addition mechanism, and conjugation with an amine produced a pyrrole derivative, analogously to the work by Peterson.\(^53\) To establish the same reactivity in DNA, ensuring that oligonucleotide incorporation did not affect reactivity observed during nucleoside studies, the 3-furyl-guanine base was incorporated into positions G\(_5\), G\(_6\) and G\(_8\) of TBA. The MS spectra for these strands are shown in Appendix B. To fully assess the impact of these adducts on the folding ability of the TBA aptamer into its quadruplex structure, \(T_m\) values were acquired for the different strands with K\(^+\), Na\(^+\) NH\(_4\)^+ as counter ions.
Table 4-3: $T_m$ studies for 3furG-TBA strands inserted at various positions in three different buffers.

<table>
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<tr>
<th>Strand</th>
<th>Position</th>
<th>Counter Ion</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$ (°C)</th>
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<tr>
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<td></td>
<td>Na$^+$</td>
<td>24.0</td>
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<td>K$^+$</td>
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<td></td>
<td></td>
<td>NH$_4^+$</td>
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<td>-</td>
</tr>
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<td>17.3</td>
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<td></td>
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<td></td>
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</table>

Table 4-3 shows the results of these experiments. Modification at position 5 produced a stabilizing influence with all three counter ions ($\text{Na}^+ \Delta T_m = +18.3$ °C, $\text{K}^+ \Delta T_m = +8.5$ °C and $\text{NH}_4^+ \Delta T_m = +17.3$ °C). This was expected as C8-substituted guanine bases that demonstrate a syn-preference have been shown to stabilize quadruplexes when inserted at a site that requires syn orientation within the quadruplex.\textsuperscript{80,45} Position $G_6$ showed a large destabilizing influence ($\Delta T_m = -18.6$ °C). This result was attributed to the anti-orientation requirement for $G_6$ and the extra energetic cost associated with forcing the modified base into an energetically unfavorable orientation.\textsuperscript{45} At position $G_8$, the modification is located in the T-G-T loop and shows a small destabilizing influence, although the $T_m = 44.9$ °C still represents a relatively stable quadruplex structure. Although the steric influence of the aryl ring destabilizes the secondary structure slightly, the modification is still well accommodated into proper folding with a K$^+$ counter ion. Molecular dynamic simulations have shown that unmodified $G_8$ prefers an anti-conformation while C8-aryl-dG bases prefer a syn orientation.\textsuperscript{45} Although there is a small
energetic cost associated with altering the conformation of the nucleobase, the less stringent environment of the loop produces a less pronounced effect.

When comparing the counter ions used, \( T_m \)'s of the unmodified TBA show that \( K^+ \) produces the most stable quadruplex by quite a significant margin (\( T_m \) \( K^+ = 53.3 \) °C, \( T_m \) \( Na^+ = 24.0 \) °C, \( T_m \) \( NH_4^+ = 21.7 \) °C). This result supports literature findings that \( K^+ \) significantly stabilizes G-quadruplex folding. Furthermore, the destabilizing effect of \( Na^+ \) and \( NH_4^+ \) was also observed when comparing \( \Delta T_m \) of the modified strands as \( G_6 \) and \( G_8 \) did not produce calculable \( \Delta T_m \) (Table 4-3). When examining modification at \( G_5 \), it is significant to note that \( \Delta T_m \) values were much higher for \( Na^+ \) and \( NH_4^+ \) counter ions compared to \( K^+ \). This effect was attributed to the smaller ionic radius of \( Na^+ \) and \( NH_4^+ \) compared to \( K^+ \) which diminished steric interactions between the aryl moiety and the counter ions and increased the relative stability.

Circular dichroism (CD) data is shown in Figure 4-17 for the folded secondary structures of TBA aptamer in \( K_2PO_4 \) buffer with 3-furyl modification at position \( G_5 \) (red trace), \( G_6 \) (black trace), and \( G_8 \) (blue trace). The traces show a major positive CD peak ~ 290 nm, with additional positive peaks at 250 nm and 210 nm, and a negative CD peak ~ 260 nm. This pattern is characteristic of an anti-parallel G-tetrad stacking arrangement in the quadruplex, which is the known secondary structure of the TBA aptamer. Hyperchromicity observed with modifications at \( G_5 \) and \( G_8 \), compared to \( G_6 \), indicate a more ordered secondary structure with increased base-stacking interactions and a tighter quadruplex folding arrangement. This effect is attributed to more facile accommodation of the modification within a quadruplex at \( G_5 \) and \( G_8 \) and mirrors the \( T_m \) data for quadruplex stability. The modification at \( G_6 \) (black trace) shows marked hypochromicity compared to \( G_5 \) and \( G_8 \), and the most destabilizing \( T_m \) (\( \Delta T_m = -18.3 \) °C).

Based on \( T_m \) experiments and CD data, it was decided that modification at \( G_6 \) would be
too destabilizing to allow proper quadruplex folding, thus making modification at G₆ a poor candidate to continue with oxidation experiments. Furthermore, data on the TBA aptamer-thrombin interaction does not predict any close association between the protein and G₆.⁴² Therefore, oxidation studies were conducted on TBA modified at G₅ and G₈.

![Figure 4 - 17: Overlay of CD spectra for 3furG-TBA strands modified at G₅ (red trace), G₆ (black trace) and G₈ (blue trace).](image)

Oxidation experiments were performed on the modified TBA (mTBA) by making a solution of DNA to a concentration of 10 µM in Na₂PO₄. The buffer used contained sodium as the counter ion, and the reaction was done at elevated temperatures (45 °C). Based on Tₘ data (Table 4-3), these conditions ensured that the strand would remain unfolded throughout the oxidation process. Four equiv of NBS were added to the reaction mixture over 30 min while monitoring by HPLC. Although the traces were poorly resolved, the characteristic absorbance of the 1,4-dialdehyde allowed confirmation of reaction progress though the evolution of a new peak (Figure 4-18B). NAC (40 equiv) and NAL (40 equiv) were added simultaneously upon oxidation completion, and the reaction was allowed to stir overnight. A sample was removed for HPLC analysis, and although retention times of peaks had shifted, unequivocal identification could not be
resolved by HPLC analysis alone (Figure 4-18C). Addition of 10% formic acid, was followed by heating the reaction at 70 °C for 2 h to hydrolyze the DNA. Upon reinjection, a single peak co-eluted with the analytical standard (Figure 4-18 D). UV-vis, fluorescence and MS analysis confirmed the identity of the conjugate (6), corroborating the nucleoside studies. Also, the calibration curve was used to quantify successful reaction products from oxidation at each site. These results, shown in Table 4-4, indicated that modification at G₅ afforded more conjugated pyrrole adduct after NBS oxidation (78%) than G₈ (59%).

Table 4 - 4: Reaction Yields from NBS oxidation of 3fur-G modified TBA strands at position G₅ and G₈

<table>
<thead>
<tr>
<th>Modification Site</th>
<th>Areaᵃ (a.u.)</th>
<th>Concentration (µM)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₅</td>
<td>223.4</td>
<td>7.43</td>
<td>78</td>
</tr>
<tr>
<td>G₈</td>
<td>171.2</td>
<td>5.69</td>
<td>59</td>
</tr>
</tbody>
</table>

ᵃCalculated with HPLC software by integrating product peaks. ᵇDerived from calibration curve constructed for adduct (6)

Figure 4 - 18: HPLC showing oxidation and trapping studies using 3fur-TBA modified at G₅. A) Starting strand. B) 4 equiv of NBS added over 30 min. C) NAC NAL added simultaneously with stirring at 45 °C for 14 h. D) Treatment with 10% formic acid at 70 °C for 2 h. E) NAC-NAL-3fur-G (6) standard.
Interestingly, both strands were oxidized in their pre-folded state suggesting that the reactivity difference was not attributable to positional or spatial effects as both modification sites should have given comparable access to the oxidant. To explain the difference, it was useful to look instead on sequence specificity, as it is known that oxidation potentials for guanine are influenced by neighboring base sequences. Experimental studies have shown that sequences with repeated G bases show greater propensity to oxidize than solitary G’s which has been attributed to formation of delocalized hole traps. The TBA sequence (5'-GGTTG6G6TG6TGTTGG-3') shows that G5 is flanked by a G and a T, while G8 is flanked by two T’s. DFT calculations illustrated that sequence effects were significant when examining the oxidation potential of a guanine flanked by two bases. Guanine in a TGG triplet has a lower ionization potential than guanine in a TGT triplet (6.62 eV vs 6.96 eV). Furthermore, the base on the 3’ side of guanine has a greater influence on oxidation potential. This sequence specificity could explain why oxidation at G5 produced more product than oxidation reactions at G8.

4.3.4. Protein Trapping Experiments with 3-furyl modified TBA

Chemical reactivity of mTBA showed promise for covalent applications and provided proof-of-concept for this oxidation system. Ultimately, it was desirable to demonstrate covalent reactivity between the aptamer and its target, thrombin. To accomplish this, modified mTBA with 3-furyl-G at G5 and G8 were oxidized with NBS in Na2PO4 buffer. Upon completion of oxidation, 40 equiv NAC and 1 equiv bovine thrombin were added while stirring at 20 °C. The reactions were stirred overnight and reaction products were analyzed by polyacrylamide gel electrophoresis (PAGE, Figure 4-19).
Figure 4 - 19: PAGE gel stained with Coomassie blue showing modified TBA oxidation cross-linking reactions. Well designations: 1) Protein ladder, 2) BSA, 3) Bovin thrombin protein 4-6) 3-furyl modified TBA at G_8 7-9) 3-furyl modified TBA at G_5.

Wells 4-6 show increasing concentrations of 3-furyl modified TBA at G_8, while wells 7-9 show increasing concentrations of 3-furyl modified TBA at G_5. Wells 5 and 6 showed a distinct band that ran slightly slower than thrombin (Figure 4-19, arrows). In contrast, wells 7-9 failed to show the anticipated band for trapping at position G_5 (Figure 4-19, wells 7-9). The gel results are, therefore, suggestive of a covalent reaction between TBA and thrombin when the oxidized 3-furyl modification is at G_8. Comparing the band to the ladder, the mass of the higher band corresponds to the mass of TBA and thrombin. Although further studies are required to confirm the identity of the band ascribed to cross-linked material, these preliminary results were encouraging and appeared to show site specific interaction between mTBA and the thrombin target.

Previous studies containing crystallographic evidence of the precise interactions between TBA and thrombin were analyzed to explain the observed site specificity of the trapping reaction. Borbone and colleagues have studied the effects of modification on T_7 and T_12 of TBA to enhance binding stability. Their crystallographic work of the TBA-
thrombin complex showed close interactions of G₈ with Arg 75, Lys 78, and Lys 70 while no associations were observed with G₅.⁴² The quadruplex nucleobases did not show close association with the protein upon binding, and the lack of access to an amine nucleophile may explain why no covalent reaction was observed at G₅. Figure 4-21 shows the aptamer protein complex with G₈ highlighted in yellow illustrating this close contact.

![Thrombin protein complex with G₈ highlighted in yellow](image)

**Figure 4 - 20**: TBA-thrombin binding complex taken from crystallography file HAL1 showing G₈ in yellow

### 4.4. Conclusions.

Efforts to use established chemical means to confer covalent functionality to an aptamer oligonucleotide were attempted, and reported in Chapter 4. The goal of this project was to provide proof-of-concept for the use of furyl modifications to covalently link an aptamer to its protein target. The extensive knowledge on TBA allowed for intelligent modification site specifically, and the reactivity was tested in a step-wise manner starting with the nucleoside. Both the 2-furyl and 3-furyl-C8-modified dG adducts were oxidized, and demonstrated the desired ring-opening oxidation pathway to form the reactive 1,4-butene-dial adduct. Due to competitive bromination observed upon oxidation of 2-fur-dG, the 3-furyl modified base was selected for further experimentation in TBA, due to its more selective and efficient reactivity.
Covalent trapping of the nucleoside using a sulphur nucleophile (NAC) and pyrrole formation upon addition of a nucleophilic amine (NAL) was successful and mimicked the reaction pathway established by Peterson and co-workers.\textsuperscript{75} Reactivity proceeded analogously in TBA, and trapped products were isolated and quantified with the use of the fully characterized pyrrole adduct standard. Rose bengal was used to examine the effect of singlet oxygen on this system and also produced the pyrrole adduct. Finally, the system was implemented with the aptamer and the target protein. The results of these experiments suggested successful covalent reactivity, evidenced by gel electrophoresis. This observation was supported by crystallographic experiments that show a close interaction between TBA nucleobases on the T\textsubscript{7}-G\textsubscript{8}-T\textsubscript{9} loop, and reactive amino acids (Lys and Arg), which may explain selectivity for cross-linking.

As there are only two nucleobases in TBA, modification of thymine residues to expand upon potential reaction sites for covalent target trapping of thrombin, would be of great interest for future work in this area. Also, the DNA-protein adduct must be fully characterized to ensure that the bands observed in the gel correspond to the protein-aptamer conjugate. Finally, it would be advantageous to explore furan derivatives that could be photo-oxidized and ring opened without the addition of extra oxidant species. This would expand the applications of this technique to a wider range of biological targets. The work reported in Chapter 4 provides a foundation to further explore oxidative reactivity in a wide range of aptamers. The furyl modification is small, does not greatly perturb secondary folding of aptamers, and can be selectively oxidized to produce a highly reactive electrophilic site capable of binding nucleophilic amino acids, linking the aptamer to its target. This work should aid in the development of aptamer tools for future research in target identification and aptamer therapeutic enhancements.
4.5. References


Appendix A

General Experimental Procedures.
A.1. General Chemical Details

Unless specifically noted, all commercial compounds were used as received. Anhydrous 1,4-dioxane, \( N,N \)-dimethylformamidyl diethyl acetal and 4,4-dimethoxytriphenylmethyl chloride (DMT-Cl) were purchased from Sigma-Aldrich (Oakville, ON). Xylenes, pyridine, and methylene chloride were purchased from Fisher Scientific (Ottawa, ON), distilled over CaH\(_2\), and stored under nitrogen. 2′-Deoxyguanosine (dG) monohydrate was purchased from ChemGenes (Wilmington, MA). All boronic acids were purchased from Frontier Scientific (Logan, UT). Dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from an LC Technology SP-105 solvent purification system at the University of Guelph. Triethylamine (TEA) was distilled from NaH. Trisphenylphosphate-3,3′,3″-trisulfonic acid trisodium salt hydrate (TPPTS), \( N_\alpha \)-Acetyl-L-lysine (NAL) and \( N \)-acetylcysteine (NAC) were purchased from Alpha Aesar (Ward Hill, MA). 2-Cyanoethyl-\( N,N \)-diisopropyl-chlorophosphoramidite was purchased from ChemGenes. All unmodified oligonucleotides were purchased from Sigma-Aldrich, including \( Narl \), 5′-CTCGGCCTCATC-3′, \( Narl \) (\( N = C \)), 5′-GATGGCGCCAG-3′, \( Narl \) (\( N = G \)), 5′-GATGGGGCCAG-3′, \( Narl \) (\( N = -2 \)), 5′-GATGGGCCAG-3′ and \( Narl \) (\( N = \) THF), 5′-GATGG-THF-GCCAG-3′. Concentration and purity were determined by UV-vis spectroscopy. Triethylammonium acetate (TEAA) buffer was prepared from glacial acetic acid and triethylamine. Water used for buffers and spectroscopic solutions was obtained from a Milli-Q filtration system at 18.2 MΩ.

A.2. Nuclear Magnetic Resonance (NMR)

\(^1\)H and \(^{13}\)C and \(^{31}\)P nuclear magnetic resonance (NMR) spectra were recorded on a NMR spectra were recorded on a Bruker Avance DPX 300, 400, or 600 MHz spectrometers in either DMSO-\( d_6 \), CDCl\(_3\) or acetone-\( d_6 \) at the NMR facility (University of
Two dimensional (2D) NMR experiments with water suppression were performed on a Bruker Avance 600 MHz spectrometers by Valerie Robertson and Dr. Andy Lo. All NMR spectra were referenced to the residual proton signal on the deuterated solvent and processed using TopSpin software versions 2.1 and 3.1. All experiments were carried out at 298 K.

A.3. Spectroscopy (UV-vis, Fluorescence)

UV-vis spectra for quantification and characterization of oligonucleotides, as well as thermal melting curves and photophysical measurements were acquired on a Cary 300-Bio UV-Visible spectrophotometer equipped with a Peltier block-heating unit and automated temperature controller. Standard 10 mm light path quartz glass cells from Hellma GmbH&Co were used for quantification, while Hellma 114-QS 10 mm light path cells were used for thermal melting experiments. CD spectra were obtained on a Jasco J-815 CD Spectrophotometer, equipped with a 1 x 6 Multicell Block Peltier, thermal controller and Julabo AWC 100 water circulator unit. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence Spectrophotometer equipped with a 1 x 4 Multicell Block Peltier, stirrer and temperature controller with Probe series II.

A.4. Circular Dichroism (CD)

CD spectra were recorded using 110-QS cells with a light path of 1 mm. A Denver Instrument UB-10 pH meter was used for buffer calibration. Calibration of the pH meter was carried out using buffers purchased from Fisher at pH 2.00, 4.00 and 7.00.

A.5. Chromatography

Separation and purification of synthetic products was achieved using standard normal phase column chromatographic methods, with silica as the stationary phase.
Columns were loaded wet after solvent equilibration and air pressure was used to elute products.

HPLC purification was performed on an Agilent 1200 series purification system equipped with a 1200 series diode array and multiple wavelength detector. The column used for HPLC purification of oligonucleotides was a Phenomenex clarity 5 μ oligo-RP semi-preparative column. Analytical oligonucleotide HPLC purification was performed on a Phenomenex Clarity 3μ Oligo 50 x 4.60 mm 3 micron column. Analytical HPLC purification of nucleoside adduct work was conducted on an Agilent PLRP-S column 3 μm 100 Å column.

A.6. Mass Spectrometry

High resolution Mass spectrometry was conducted at the Biological Mass Spectrometry Facility at the University of Guelph by Dr. Dyanne Brewer and Dr. Armen Charchoglyan. Spectra were acquired on a Agilent UHD 6530 Q-Tof mass spectrometer operating in nanospray ionization at 0.5 µL/min detecting positive ions. Low resolution Mass Spectrometric analysis was obtained on a Bruker AmaZon SL spectrometer equipped with an ion trap through direct injection.
Appendix B.

Mass Spectrometry Data.
Table B - 1. MS Analysis of $^{NaphO}dG$-Modified *Narl* Oligonucleotides.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass</th>
<th>exptl $m/z$ (ESI$^+$)$^a$</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Narl</em>($X = ^{NaphO}dG$)</td>
<td>$C_{124}H_{153}N_{42}O_{72}P_{11}$</td>
<td>3722.7</td>
<td>[M − 4H]$^4−$ = 929.8</td>
<td>3723.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 5H]$^5−$ = 743.6</td>
<td>3723.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 6H]$^6−$ = 619.5</td>
<td>3723.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]$^7−$ = 530.9</td>
<td>3723.3</td>
</tr>
</tbody>
</table>

**Figure B - 1. MS Spectrum for $^{NaphO}G$ modified *Narl* 12mer sequence at the G$_3$ position.**
Table B - 2. MS Analysis of PhPhO\textsubscript{dG}-Modified Narl Oligonucleotide.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass$^a$</th>
<th>exptl m/z (ESI$^-$)$^b$</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narl($X = \text{PhPhO}_{dG}$)</td>
<td>$C_{126}H_{156}N_{42}O_{72}P_{11}$</td>
<td>3748.7</td>
<td>[M − 4H]$^{4−} = 936.3$</td>
<td>3748.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 5H]$^{5−} = 748.7$</td>
<td>3748.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 6H]$^{6−} = 623.8$</td>
<td>3748.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]$^{7−} = 534.3$</td>
<td>3749.2</td>
</tr>
</tbody>
</table>

Figure B - 2. MS Spectrum for PhPhO\textsubscript{dG} modified Narl 12mer sequence at the G\textsubscript{3} position.
Table B - 3. MS Analysis of \( ^{\text{CNBP}} \text{dG} \)-Modified \( \text{Nar} \) Oligonucleotide.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass(^a)</th>
<th>exptl ( m/z ) (ESI)(^b)</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narl(( X = ^{\text{CNBP}} \text{dG} ))</td>
<td>( \text{C}<em>{127}\text{H}</em>{154}\text{N}<em>{43}\text{O}</em>{72}\text{P}_{11} )</td>
<td>3773.6</td>
<td>[( M - 4H )](^+) = 942.5</td>
<td>3774.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[( M - 5H )](^+) = 753.8</td>
<td>3774.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[( M - 6H )](^+) = 627.9</td>
<td>3773.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[( M - 7H )](^+) = 538.1</td>
<td>3773.7</td>
</tr>
</tbody>
</table>

Figure B - 3. MS Spectrum for \( ^{\text{CNBP}} \text{G} \) modified \( \text{Nar} \) 12mer sequence at the \( G_3 \) position.
Table B - 4. MS Analysis of TCPO-dG-Modified Narl Oligonucleotide.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass</th>
<th>exptl m/z (ESI)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narl (X = TCPO-dG)</td>
<td>C&lt;sub&gt;120&lt;/sub&gt;H&lt;sub&gt;148&lt;/sub&gt;N&lt;sub&gt;42&lt;/sub&gt;Cl&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;22&lt;/sub&gt;P&lt;sub&gt;11&lt;/sub&gt;</td>
<td>3777.7</td>
<td>[M − 5H]&lt;sup&gt;b&lt;/sup&gt; = 754.5</td>
<td>3777.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 6H]&lt;sup&gt;b&lt;/sup&gt; = 628.5</td>
<td>3777.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]&lt;sup&gt;b&lt;/sup&gt; = 538.7</td>
<td>3777.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 8H]&lt;sup&gt;b&lt;/sup&gt; = 471.2</td>
<td>3777.6</td>
</tr>
</tbody>
</table>

Figure B - 4. MS Spectrum for TCPOG modified Narl 12mer sequence at the G<sub>3</sub> position.
Figure B - 5. Oligonucleotide fragment-ion nomenclature proposed by McLuckey.

Figure B - 6. Nomenclature of major fragments from oligonucleotide fragmentation at the $O$-linked modified site.
Table B - 5. MS Analysis of \( {\text{PCPO}} \)-Modified Narl Oligonucleotide.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass</th>
<th>exptl ( m/z ) (ESI(^−))^{a}</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narl (X=( {\text{PCPO}} )G)</td>
<td>( \text{C}<em>{120}\text{H}</em>{146}\text{Cl}<em>{5}\text{N}</em>{42}\text{O}<em>{72}\text{P}</em>{11} )</td>
<td>3842.7</td>
<td>( [\text{M}−4\text{H}]^{4−}=959.6 )</td>
<td>3842.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( [\text{M}−5\text{H}]^{5−}=767.5 )</td>
<td>3842.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( [\text{M}−6\text{H}]^{6−}=639.4 )</td>
<td>3842.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( [\text{M}−7\text{H}]^{7−}=547.9 )</td>
<td>3842.3</td>
</tr>
</tbody>
</table>

Figure B - 7. MS Spectrum for \( {\text{PCPO}} \) modified Narl 12mer sequence at the \( G_3 \) position.
Figure B - 8. MS$^2$ Spectrum for $\text{PCOP}_G$ modified NarI 12mer sequence at the $G_3$ position.
Table B - 6. MS Analysis of $^{8nO}dG$-Modified *Nar* Oligonucleotide.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>product formula</th>
<th>calcd mass</th>
<th>exptl $m/z$ (ESI)$^a$</th>
<th>exptl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nar</em> (X= $^{8nO}G$)</td>
<td>$C_{121}H_{153}N_{42}O_{72}P_{11}$</td>
<td>3687.7</td>
<td>$[M − 6H]^{6−} = 613.6$</td>
<td>3687.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$[M − 7H]^{7−} = 525.8$</td>
<td>3687.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>$[M − 8H]^{8−} = 460.0$</td>
<td>3688.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$[M − 9H]^{9−} = 408.8$</td>
<td>3688.2</td>
</tr>
</tbody>
</table>

Figure B - 9. MS Spectrum for $^{8nO}G$ modified *Nar* 12mer sequence at the G$_3$ position.
Figure B - 10. ESI⁻-MS² Spectrum for BnO-G modified Narl 12mer sequence at the G₃ position.

Figure B - 11. ESI⁻-MS³ Spectrum for BnO-G modified Narl 12mer sequence at the G₃ position.
Figure B - 52. MS Spectrum for 3’',4’’-DHPH-dG.
Table B - 7. MS Analysis of 3fur-G Modified TBA at positions G₅, G₆ and G₈.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>product formula</th>
<th>calcd mass a</th>
<th>exptl m/z (ESI) b</th>
<th>exptrl mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA (3furG at G₅)</td>
<td>C₁₅₄H₁₈₉N₅₇O₉₅P₁₄</td>
<td>4789.8</td>
<td>[M − 6H]⁺ = 797.3</td>
<td>4789.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]⁺ = 683.2</td>
<td>4789.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 8H]⁺ = 597.8</td>
<td>4790.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 9H]⁺ = 531.2</td>
<td>4789.8</td>
</tr>
<tr>
<td>TBA (3furG at G₆)</td>
<td>C₁₅₄H₁₈₉N₅₇O₉₅P₁₄</td>
<td>4789.8</td>
<td>[M − 6H]⁺ = 797.3</td>
<td>4789.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]⁺ = 683.4</td>
<td>4790.8</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 8H]⁺ = 597.7</td>
<td>4789.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 9H]⁺ = 531.3</td>
<td>4790.6</td>
</tr>
<tr>
<td>TBA (3furG at G₈)</td>
<td>C₁₅₄H₁₈₉N₅₇O₉₅P₁₄</td>
<td>4789.8</td>
<td>[M − 6H]⁺ = 797.4</td>
<td>4790.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 7H]⁺ = 683.3</td>
<td>4790.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 8H]⁺ = 597.5</td>
<td>4788.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M − 9H]⁺ = 531.3</td>
<td>4790.6</td>
</tr>
</tbody>
</table>

Figure B - 6. MS Spectrum for 3fur-G modified TBA at position G₅.
Figure B - 7. MS Spectrum for 3fur-G modified TBA at position G₆.

Figure B - 8. MS Spectrum for 3fur-G modified TBA at position G₆.
Appendix C.

NMR Spectra for Synthetic Samples.
Figure C - 1. $^1$H NMR spectrum of 8-(2-naphthoxy)-2'-deoxyguanosine (NaphO\textsuperscript{dG}) in DMSO-d$_6$. 

* = TBAF
Figure C - 2. $^{13}$C NMR spectrum of 8-(2-naphthoxy)-2'-deoxyguanosine ($^{\text{NaphO}}_{\text{dG}}$) in DMSO-$d_6$. 
Figure C - 3. $^1$H NMR spectrum of 8-(4-phenylphenoxy)-2'-deoxyguanosine ($^{PhPhO}$dG) in DMSO-$d_6$. 

* = TBAF
Figure C - 4. $^{13}$C NMR spectrum of 8-(4-phenylphenoxy)-2'-deoxyguanosine ($\text{PhPhO}^\text{dG}$) in DMSO-$d_6$. 
Figure C - 5. $^1$H NMR spectrum of 8-(4-cyanophenylphenoxy)-2'-deoxyguanosine (CNBPOdG) in DMSO-d$_6$. 

* = TBAF
Figure C - 6. $^{13}$C NMR spectrum of 8-(4-cyanophenylphenoxy)-2'-deoxyguanosine ($^{CNBPO}$dG) in DMSO-$d_6$. 
Figure C - 7. $^1$H NMR of 8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine ($^{TCPO}$dG) in DMSO-$d_6$. 

*[TBAF]*

Current Data Parameters
NAME aw-2013-06-13
EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
Date_ 20130613
Time 21.02
INSTRUM av300
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 80
DS 0
SWH 4789.272 Hz
FIDRES 0.146157 Hz
AQ 3.4209793 sec
RG 574.7
DW 104.400 usec
DE 6.00 usec
TE 298.7 K
DI 1.0000000 sec
TDO 1

======== CHANNEL f1 ========
NUC1 1H
P1 13.50 usec
PL1 0 dB
PL1W 10.29873466 W
SF01 300.1317168 MHz

F2 - Processing parameters
SI 32768
SF 300.1300074 MHz
WDW EM
SSB 0
LB 0.10 Hz
GB 0
PC 1.00
Figure C - $^13$C NMR of 8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine ($^{13}$TCPD$_2$G) in DMSO-$d_6$. 
Figure C - $^1$H NMR of 8-(pentachlorophenoxy)-2'-deoxyguanosine (PCPO\textsuperscript{d}G) in DMSO-d\textsubscript{6}.
Figure C - 13C NMR of 8-(pentachlorophenoxy)-2'-deoxyguanosine (PCPO dG) in DMSO-d6.
Figure C - 11. $^1$H NMR of 8-(benzyloxy)-2'-deoxyguanosine (8\textsuperscript{Bn}dG) in DMSO-d$_6$. 
Figure C - 13C NMR of 8-(benzyloxy)-2'-deoxyguanosine ($^{13}$BO$_2$G) in DMSO-d$_6$. 
Figure C - 1H NMR of $N^2$-(Dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine in CDCl₃.
Figure C - 13C NMR of $N^2$-[(Dimethylformamidyl)]-8-(2-naphthoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 15. $^1$H NMR of N$^2$-(Dimethylformamidy)-8-(4-phenylphenoxy)-2'-deoxyguanosine in DMSO-d$_6$. 

*= TBAF

Current Data Parameters
NAME FB-2013-05-03-DMF(H)
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date 20130503
T1mc 16.10
INSTRUM spect
PROBHD 5 mm CPBBBO BB
PULPROG zg30
TD 44774
SOLVENT DMSO
NS 4
DS 0
SWH 5597.015 Hz
FIDRES 0.125006 Hz
AQ 3.9998107 sec
RG 181
DW 89.333 usec
DE 10.00 usec
TE 296.0 K
DI 2.00000000 sec
TD0 1

--------- CHANNEL f1 ---------
SFO1 400.1324708 MHz
NUC1 1H
P1 11.60 usec
PLW1 11.00000000 W

F2 - Processing parameters
SI 65536
SF 400.1300082 MHz
WDW EM
SSB 0
LB 0.10 Hz
GB 0
PC 1.00
Figure C - 13C NMR of N²-(Dimethylformamidyl)-8-(4-phenylphenoxy)-2'-deoxyguanosine in DMSO-d₆.
Figure C - 1H NMR of $N^2$-(Dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - $^{13}$C NMR of $N^\beta$-(Dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 19. $^1$H NMR of $N^\alpha$-(Dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine in DMSO-d$_6$. 

Current Data Parameters

NAME      aw_2013_03_14
EXPNO     1
PROCNO    1

F2 - Acquisition Parameters
Date_  20130314
Time_  13.56
INSTRUM_ spect
PROBHD  5 mm CPTCI 1H-
PULPROG  zg30
TD_  65536
SOLVENT_ DMSO
NS_  4
DS_  0
SWH_  8417.509 Hz
FIDRES_ 0.128441 Hz
AQ_  3.8928385 sec
RG_  28.5
DW_  59.400 usec
DE_  20.00 usec
TE_  295.0 K
D1_  1.000000000 sec
TD0_  1

======== CHANNEL f1 ========
NUC1_ 1H
P1_ 9.00 usec
PL1_ 4.90 dB
PL1W_ 8.12830544 W
SF01_ 600.1037061 MHz

F2 - Processing parameters
SI_ 32768
SF_ 600.1000164 MHz
WDW_ EM
SSB_ 0
LB_ 0.30 Hz
GB_ 0
PC_ 1.40
Figure C - 20. $^{13}$C NMR of $N^2$-(Dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine in DMSO-d$_6$. 
Figure C - 21. $^1$H NMR of $N^2$-(Dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in DMSO-d$_6$. 
Figure C - 22. $^{13}$C NMR of $N^\circ$-(Dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in DMSO-$d_6$. 
Figure C - 23. $^1$H NMR of $N^\delta$-(Dimethylformamidyl)-8-(benzyloxy)-2'-deoxyguanosine in DMSO-d$_6$. 

Current Data Parameters
NAME      AW_2013_04_20
EXPNO                 2
PROCNO                1

F2 - Acquisition Parameters
Date_          20130420
Time              13.28
INSTRUM           spect
PROBHD   5 mm CPTCI 1H-
PULPROG            zg30
TD                65536
SOLVENT            DMSO
NS                    4
DS                    0
SWH            8417.509 Hz
FIDRES        0.128441 Hz
AQ            3.8928385 sec
RG                   32
DW               59.400 usec
DE                20.00 usec
TE                295.1 K
D1           1.00000000 sec
TD0                   1

======== CHANNEL f1 ========
NUC1                 1H
P1                 9.00 usec
PL1                4.90 dB
PL1W         8.12830544 W
SFO1        600.1037061 MHz

F2 - Processing parameters
SI                32768
SF      600.1000201 MHz
WDW                  EM
SSB      0
LB                 0.30 Hz
GB       0
PC                 1.00
Figure C - 13C NMR of N²-(Dimethylformamidyl)-8-(benzylxyloxy)-2'-deoxyguanosine in DMSO-d₆.
Figure C - 1H NMR of 5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-N2-(dimethylformamidyl)-8-(2-naphthoxy)-2ꞌ-deoxyguanosine in CDCl₃.
Figure C - 26. $^{13}$C NMR of 5'-O-(4,4'-Dimethoxytrityl)-$\text{N}^3$-(dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C-27. $^1$H NMR of 5'-O-(4,4'-Dimethoxytrityl)-N'(dimethylformamidyl)-8-(4-phenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 

```
1.018
1.006
2.158
4.353
3.763
11.270
4.793
1.000
0.281
1.048
1.151
6.789
1.358
1.419
7.148
1.146
```

---

**Current Data Parameters**

Date: 2013-08-13

Time: 16:06

**Instruments**

**Solvent**: CDCl$_3$

**Sample**: 6.5536

**Temperature**: 295.0 K

**Total Acquisition Parameters**

**Probing**: 5 mm CPT-1B

**Detector**: T852

**Tuned**: 641.700 Hz

**Acq**: 0.12841 sec

**Acq**: 59,113 sec

**FID**: 3.8264 Hz

** Acquisition**: 5,000 sec

**Processing Parameters**

**Frequency**: 600.1000 MHz

**Sweep**: 0.10 sec

**Time**: 1,000.00 sec
Figure C - $^{13}$C NMR of 5'-O-(4,4'-Dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(4-phenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 29. $^1$H NMR of 5'-O-(4,4'-Dimethoxytrityl)-$N^\beta$-(dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 30. $^{13}$C NMR of 5'-O-(4,4'-Dimethoxytrityl)\(-N^2\)-\(\text{N}^2\)-\(\text{N}^2\)-(dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 31. $^1$H NMR of 5'-O-(4,4'-Dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine in CDCl$_3$. 

Figure C - 32. $^{13}$C NMR of 5'-O-(4,4'-Dimethoxytrityl)-$N^2$-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 33. $^1$H NMR of 5'-O-(4,4'-Dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in CDCl$_3$. 
Figure C - 13C NMR of 5'-O-(4,4'-Dimethoxytrityl)-N2-(dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in CDCl3.
Figure C - 35. $^1$H NMR of 5'-O-(4,4'-Dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(benzyloxy)-2'-deoxyguanosine in DMSO-d$_6$. 
Figure C - 36. $^{13}$C NMR of 5ꞌ-O-(4,4ꞌ-Dimethoxytrityl)-$N^\theta$-(dimethylformamidyl)-8-(benzoyloxy)-2ꞌ-deoxyguanosine in DMSO-$d_6$. 

$^{13}$C NMR spectrum of a compound showing carbon chemical shifts.
Figure C - 37. $^1$H NMR of 3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine in Acetone-d$_6$. 

Current Data Parameters
NAME     aw-2013-08-16
EXPNO    1
PROCNO   1

F2 - Acquisition Parameters
Date_ 20130816
Time_ 22:11
INSTRUM Spect
PROBHD 5 mm PATXO 19F
PULPROG zg30
TD 65536
SOLVENT Acetone
NS 16
DS 0
SWH 7812.500 Hz
FTDRS 0.119209 Hz
AQ 4.1943040 sec
RG 203
DW 64.000 usec
DE 6.50 usec
TE 297.1 K
DI 2.00000000 sec
TD0 1

====== CHANNEL f1 ======
SFO1 600.133608 MHz
NUC1 1H
F1 11.00 usec
PLW1 30.00000000 W

F2 - Processing parameters
SI 65536
ISF 600.1300553 MHz
WDW EM
SSB 0
LB 0.10 Hz
GB 0
PC 5.00
Figure C - 31P NMR of 3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N²-(dimethylformamidyl)-8-(2-naphthoxy)-2'-deoxyguanosine in Acetone-d₆.
Figure C - 39. $^1$H NMR of 3'-O-[(2-Cyanoethoxy)(disopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N^2-(dimethylformamidyl)-8-(4-phenylphenoxy)-2'-deoxyguanosine in Acetone-$d_6$. 
Figure C - 40. $^{31}$P NMR of 3′-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5′-O-(4,4′-dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(4-phenylphenoxy)-2′-deoxyguanosine in Acetone-d$_6$. 

Current Data Parameters
NAME aw_2013_08_14
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date 20130814
Time 17.01
INSTRUM av300
PROBHD 5 mm PABBO BB-
PULPROG zgcd
TD 32768
SOLVENT Acetone
NS 64
DS 0
SWH 40160.641 Hz
FTDRES 1.225605 Hz
AQ 0.4079616 sec
RG 14596.5
DW 12.450 usec
DE 6.00 usec
TE 295.8 K
DI 1.0000000 sec
dil 0.0300000 sec
TD0 1
SFO1 121.5051631 MHz
NUC1 31P
P1 7.20 usec
PLW1 -1.00000000 W
SFO2 300.1318044 MHz
NUC2 1H
CPDPRG[2] waltz16
PLW2 -1.00000000 W
PLW12 -1.00000000 W

F2 - Processing parameters
SI 32768
SF 121.4948360 MHz
WDW EM
SSB 0
LB 5.00 Hz
GB 0
PC 1.40
Figure C - 41. $^1$H NMR of 3’-$\text{-O-}$(2-Cyanoethoxy)(diisopropylamino)phosphino]-5’-$\text{-O-}$(4,4’-dimethoxytrityl)-$^N$-$\text{(dimethylformamidyl)}$]-8-$\text{-}$(4-cyanophenylphenoxy)-2’-deoxyguanosine in CDCl$_3$. 

$^*$ = TEA
Figure C - $^{31}$P NMR of 3’-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5’-O-(4,4’-dimethoxytrityl)-N²-(dimethylformamidyl)-8-(4-cyanophenylphenoxy)-2’-deoxyguanosine in CDCl₃.
Figure C - 43. $^1$H NMR of 3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-$^\text{N}^2$-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2'-deoxyguanosine in Acetone-d$_6$. 
Figure C - $^{31}$P NMR of 3’-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5’-O-(4,4’-dimethoxytrityl)-$N^\prime$-(dimethylformamidyl)-8-(2,4,6-trichlorophenoxy)-2’-deoxyguanosine in Acetone-d$_6$. 
Figure C - 1H NMR of 3'-O-[(2-Cyanoethoxy)(disopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N²-(dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in Acetone-d₆.
Figure C - $^{31}$P NMR of 3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N$^\prime$-(dimethylformamidyl)-8-(pentachlorophenoxy)-2'-deoxyguanosine in Acetone-d$_6$. 
Figure C - 47. $^1$H NMR of 3$'$-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5$'$-O-(4,4'$'$-dimethoxytrityl)-N$^\beta$- (dimethylformamidyl)-8-(benzyloxy)-2$'$-deoxyguanosine in Acetone-d$_6$.

* = TEA
Figure C - 31P NMR of 3'-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N²-(dimethylformamidyl)-8-(benzlyoxy)-2' -deoxyguanosine in Acetone-d₆.
Figure C - 49. $^1$H NMR of 8-Bromo-2'-deoxyguanosine (8-Br-dG) in DMSO-d$_6$. 

Current Data Parameters
NAME   aw-2014-02-18
EXPNNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_   20140218
Time_   16.47
INSTRUM  spect
PROBHD  5 mm CPTCI 1H-
PULPROG  zg30
TD      65536
SOLVENT DMSO
NS      4
DS      0
SWH     8417.509 Hz
FIDRES  0.128441 Hz
AQ      3.8928385 sec
RG      12.7
DW      59.400 usec
DE      20.00 usec
TE      295.1 K
D1      1.00000000 sec
TDO     1

====== CHANNEL f1 ======
NUC1  1H
F1    8.60 usec
PL1   5.30 dB
PL1W  7.41310215 W
SF01  600.0037054 MHz

F2 - Processing parameters
SI     32768
SF     600.0000213 MHz
WDW    EM
SSB    0
LB     0.10 Hz
GB     0
PC     1.00
Figure C - $^{13}$C NMR of 8-Bromo-2'-deoxyguanosine (8-Br-dG) in DMSO-d$_6$. 
Figure C - 51. $^1$H NMR of 8-(4"-hydroxyphenyl)-2'-deoxyguanosine (p-PhOH-dG) in DMSO-d$_6$. 

```
Current Data Parameters
NAME  aw-2014-02-18
EXPNO 3
RCCNO 1

F2 - Acquisition Parameters
Date  20140218
Time  17.02
INSTRUM spect
PROBHD 5 mm CPTCI 1H-
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 4
DS 0
SWH 8417.509 Hz
FITRES 0.128441 Hz
AQ 3.8928385 sec
RG 16
DW 59.400 usec
DE 20.00 usec
TE 295.0 K
DI 1.00000000 sec
TDO 1

CHANNEL f1
NUC1 1H
F1 8.60 usec
PL1 5.30 dB
PL1W 7.41310215 W
SF1 600.0037654 MHz

F2 - Processing parameters
SI 32768
SF 600.0000104 MHz
WDW EM
SSS 0
LB 0.10 Hz
GB 0
PC 1.00
```
Figure C - $^{13}$C NMR of 8-(4''-hydroxyphenyl)-2'-deoxyguanosine (p-PhOH-dG) in DMSO-d$_6$. 
Figure C - 53. $^1$H NMR of 8-(3''',4'''-dihydroxyphenyl)-2'-deoxyguanosine (3''',4'''-DHP-dG) in DMSO-d$_6$. 
Figure C - 13C NMR of 8-(3\''\',4\''\'-dihydroxyphenyl)-2\'-deoxyguanosine (3\'',4\''-DHP-dG) in DMSO-d6.
Figure C - 55. $^1$H NMR of 8-(2-furyl)-2'-deoxyguanosine (2fur-dG) in DMSO-d$_6$. 
Figure C - $^{13}$C NMR of 8-(2-furyl)-2$'$-deoxyguanosine (2fur-dG) in DMSO-d$_6$. 
Figure C - 57. $^1$H NMR of 8-(3-furyl)-2$'$-deoxyguanosine (3fur-dG) in DMSO-$d_6$. 
Figure C - 58. $^{13}$C NMR of 8-(3-furyl)-2'-deoxyguanosine (3fur-dG) in DMSO-d$_6$. 
Figure C - 59. $^1$H NMR of N$^2$-(Dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine in DMSO-d$_6$. 
Figure C - 60. $^{13}$C NMR of N²-(Dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine in DMSO-d$_6$. 
Figure C - 61. $^1$H NMR of 5'-O-(4,4'-dimethoxytrityl)-N$^2$-(Dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine in Acetone-d$_6$. 
Figure C - 62. $^{13}$C NMR of 5′-O-(4,4′-dimethoxytrityl)-N$^2$-(Dimethylformamidyl)-8-(3-furyl)-2′-deoxyguanosine in Acetone-d$_6$. 
Figure C - 63. $^1$H NMR of 3'-O-[[2-Cyanoethoxy](diisopropylamino)phosphino]-5'-O-(4,4'-dimethoxytrityl)-N$^2$-(dimethylformamidyl)-8-(3-furyl)-2'-deoxyguanosine in Acetone-$d_6$. 
Figure C. \(^{31}P\) NMR of 3-O-[2-Cyanoethoxy(diisopropylamino)phosphino]-5-O-(4,4-dimethoxytrityl)-2-deoxyguanosine in Acetone-\(d_6\).
Figure C - 65. $^1$H NMR of NAC-NAL-3fur-G adduct in DMSO-$d_6$ with water suppression.
Figure C - 66. 2D COSY NMR of NAC-NAL-3fur-G adduct in DMSO-\textit{d}_6 with water suppression.
Figure C - 67. 2D HSQC NMR of NAC-NAL-3fur-G adduct in DMSO-d$_6$ with water suppression.
Figure C - 68. 2D HMBC NMR of NAC-NAL-3fur-G adduct in DMSO-d$_6$ with water suppression.