Abstract

Diana Degen: Oxidation of 2'-Deoxyguanosine and 8-Oxo-2'-deoxyguanosine by Dimethyldioxirane (Under direction of Dr. L.M. Ball)

Oxidative damage to DNA can lead to cancer, aging, and neurological disorders. Of the four DNA bases, guanine is the most susceptible to oxidation. To help us further our knowledge of how oxidation contributes to illness and disease, it is important to study the oxidation process and products of 2'-deoxyguanosine (2'-dG). The oxidation product of 2'-dG, 8-oxo-2'-deoxyguanosine (8-oxo-2'-dG), is even more susceptible to oxidation than 2'-dG, and for this reason, it is imperative to study its oxidation products as well.

For our study, oxidations of 2'-deoxyguanosine and 8-oxo-2'-deoxyguanosine were conducted with the powerful chemical oxidant dimethyldioxirane (DMDO). Oxidation products were then characterized with the use of HPLC, proton and multidimensional heteronuclear NMR, and electrospray MS in the positive ion mode. Because DMDO reacts selectively, it was expected that oxidation would occur at all types of double bonds, and possibly at NH₂ or NH. However, it was found that the major product fraction was formed from epoxidation of the C-4 to C-5 double bond followed by solvolysis. Hence, in the reaction of DMDO with 2'-deoxyguanosine, epoxidation followed by hydrolysis or methanolysis formed 4,5-dihydro-4,5-dihydroxy-2'-deoxyguanosine and 4,5-dihydro-4-hydroxy-5-methoxy-2'-deoxyguanosine respectively. When DMDO reacted with 8-oxo-2'-deoxyguanosine in a solution of water and methanol, epoxidation followed by hydrolysis resulted in the formation of 4,5-dihydro-4,5-dihydroxy-8-oxo-2'-deoxyguanosine and epoxidation followed by methanolysis resulted in the formation of 4,5-dihydro-4-hydroxy-5-methoxy-8-oxo-2'-deoxyguanosine. The
latter was further verified by conducting oxidations in perdeuterated methanol. This research concludes that DMDO, which has an oxidation mechanism analogous to the mixed-function oxidases, generates products distinct from those arising from radical chemistry.
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Chapter 1

Introduction

DNA Oxidation

Oxidation continuously occurs in the human body through endogenous and exogenous means. Endogenous oxidant sources include: mitochondria, cytochrome P450, macrophage/inflammatory cells, and peroxisomes; whereas, exogenous oxidants include: redox cycling compounds, metals (through catalysis of the Fenton reaction), and radiation (Klaunig and Kamendulis, 2004). Oxidation can damage DNA and lead to cancer, aging, and neurological disorders (Wang et al., 1999; Beckman and Ames, 1999; and Burrows and Muller, 1998). DNA is made up of 4 bases: adenine, thymine, cytosine, and guanine. Being the most electron-rich of the bases, guanine is the easiest to oxidize (Burrows and Muller, 1998 and Steenken and Jovanovic, 1997). To help us further our knowledge of how DNA oxidation contributes to illness and disease, it is important to study the oxidation process and products of 2'-deoxyguanosine (2'-dG). Furthermore, the major characterized oxidation product of 2'-dG, 8-oxo-2'-deoxyguanosine (8-oxo-2'-dG), is even more susceptible to oxidation than is 2'-dG, and for this reason, it is imperative to study its oxidation products as well (Steenken et al., 2000).

Rigorous in vitro studies, essential in understanding the mechanism of damage and repair, are impeded by limited availability of chemically characterized synthetic 2'-dG oxidation products. Those products reported in the literature were prepared by
cumbersome procedures. In this research, dimethyldioxirane (DMDO) was used as the oxidizing agent in a mild and general procedure for the synthesis of 2'-dG and 8-oxo-2'-dG oxidation products.
**DMDO**

Dimethyldioxirane (DMDO) has many advantages as an oxidizing agent. First of all, DMDO is easily prepared from commercially available chemicals in one step as a solution in acetone. Secondly, DMDO is highly reactive, thus allowing rapid oxidation of the reactant. DMDO is also extremely useful for mimicking in vivo oxidation because it acts as a mono-oxygen donor (Edwards et al., 1987). Lastly, DMDO is a “clean” oxidizing agent because acetone is the only by-product and is easily removed after the reaction. As seen in Figure 1.1, DMDO is formed by reacting acetone with commercially available Oxone® (potassium peroxymonosulfate – H$_3$K$_5$O$_{18}$S$_4$). DMDO acts as a mono-oxygen donor and then reverts to acetone.

![Figure 1.1 Formation and reaction of DMDO](image-url)

Figure 1.1 Formation and reaction of DMDO
Chapter 2

Literature Review

DNA oxidation can be detrimental in that it can lead to DNA strand breaks, protein-DNA cross-links, abasic sites, and base lesions (Cadet et al., 1997; Henle and Linn, 1997; Demple and Harrison, 1994; and Boiteux and Radicella, 1999). If left unchecked DNA damage can lead to carcinogenesis, aging, and neurological disorders (Wang et al., 1999; Beckman and Ames, 1999; and Burrows and Muller, 1998). Therefore, DNA repair systems are essential in keeping the genome intact. It is important to define the lesions that are acted upon by repair systems. For this reason, our objective in this research is to synthesize and characterize 2'-dG oxidation products in order to possibly test them in DNA repair systems.

Previous research has established the potential for errors from DNA oxidation. For example, 8-oxo-dGTP (OG) could be formed during normal cellular metabolism in the nucleotide pool (Mo et al., 1992). If this lesion is incorporated opposite adenosine it could result in a transversion mutation G•C → T•A (Grollman et al., 1993). However, in *Escherichia coli*, two base excision repair glycosylases, Fpg and MutY, are present to prevent mutations associated with 8-oxo-dGTP by repairing OG in OG•C and OG•A respectively (David and Williams, 1998). Furthermore, *E. coli* has a third enzyme, MutT, that catalyzes hydrolysis of d(OGTP) to d(OGMP), thus preventing incorporation of d(OGTP) into the genome (Maki and Sekiguchi, 1992)
Although Fpg repairs the OG in OG•C pairs, it also has the potential to repair OG
- A mispairs, but at a much slower rate (Leipold et al., 2000). But when adenine is paired
with guanidinothydantoin (Gh) or spiroiminodihydantoin (Sp), two oxidation products of
8-oxo-guanine, this rate of removal is increased (Leipold et al., 2000). Conversely, MutY
is unable to remove A paired with Gh or Sp (Leipold et al., 2000). In another study, it
was also found that Gh and Sp are potent sources of replication errors in vivo (Henderson
et al., 2003).

Various oxidation products of 2'-deoxyguanosine and 8-oxo-2'-deoxyguanosine
have been formed through the use of singlet oxygen, one-electron oxidation, H2O2,
metals, and DMDO (Ye et al., 2003; Ravanat and Cadet, 1995; Luo et al., 2001; Suzuki et
al., 2003; Hofer, 2002; and Chworos et al., 2002). These products and their mechanisms
of formation are discussed in the next section.
**DNA Oxidation**

Singlet oxygen

Singlet oxygen (\(^1\text{O}_2\)) is formed by a process called photosensitization, in which an irradiated photosensitizer is raised to its singlet excited state and then to its triple excited state. At this point, if the photosensitizer acts directly on the target molecule it is said to undergo a Type I radical reaction. The alternative is a Type II reaction, in which the photosensitizer transfers energy to dioxygen to generate singlet oxygen (Burrows and Muller, 1998). Due to its high chemical reactivity, singlet oxygen is often used to oxidize guanine.

In one experiment using singlet oxygen (Figure 2.1) it was demonstrated that two diastereomers of 4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanosine were the major oxidation products of 2'-deoxyguanosine (Ravanat et al., 1995). The proposed mechanism involved the “initial formation of a transient endoperoxide through a Diels-Alder 1,4-cycloaddition of \(\text{O}_2\) [at \(\text{C4-C8}\) of] the purine ring” (Ravanat et al., 1995). It is then suggested that the unstable endoperoxides degrade to form two diastereomers due to the chiral center at \(\text{C4}\).

However, in a later experiment, guanidinohydantoin (Gh) and spirominodihydantoin (Sp) were identified as the major products formed from the oxidation of guanosine by singlet oxygen (Ye et al., 2001). It was proposed that singlet oxygen formed a 5-hydroxy-8-oxo-guanosine intermediate through the addition of two oxygen atoms, because both Sp and Gh each gain two oxygen atoms. It was suggested that one oxygen atom was obtained from singlet oxygen and the other was derived from the addition of \(\text{H}_2\text{O}\). Depending on pH, this unstable intermediate (5-OH-8-oxo-
guanosine) will either rearrange to give guanidinohydantoin at pH conditions below 7 or give spirominodihydantoin at pH values above 7 (Figure 2.2). In Figure 2.2, it is shown that 8-oxo-guanosine can be formed from 2 electron oxidation of guanosine and also that 5-OH-OG could be formed from a subsequent 2 electron oxidation of 8-oxo-guanosine. The 5-OH-OG oxidation intermediate differs from the 4-OH-8-oxo-guanosine formation proposed by Ravanat and Cadet. Ye et al. determined that an atom of oxygen from water was introduced at C5 of guanine by examination of the fragmenting patterns of Sp and Gh compounds with $^{18}$O incorporation. Therefore, the proposed mechanism reflects this determination and thus differs from Ravanat and Cadet as well. However, due to the instability of 5-OH-OG, it was not characterized and therefore, the structure could not be rigorously confirmed. It is also important to note that Piette found that 7,8-dihydro-8-oxo-guanine was the major $^{1}$O$_2$ oxidation product of guanine in double-stranded DNA (Piette, 1990).

H$_2$O$_2$

H$_2$O$_2$ is produced in the cell by the mitochondria, cytochrome P450, macrophage/inflammatory cells, and peroxisomes (Klaunig and Kamendulis, 2004). Therefore, H$_2$O$_2$ could be used to mimic in vivo oxidation. Hofer et al. (2001) reported the oxidation of free 2'-dG and 2'-dG in DNA by hydrogen peroxide (H$_2$O$_2$) and the reducing agent ascorbate (HAsc-) resulted in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine and guanidinohydantoin (Hofer et al., 2001). The proposed mechanism followed an initial oxidation of the C-8 position of 2'-dG and subsequent oxidation of the C-5 position to form 5-OH-8-oxo-2'-deoxyguanosine (Figure 2.3). 5-OH-8-oxo-dG is then transformed to guanidinohydantoin through the addition of H$_2$O and the loss of CO$_2$. 
Another experiment utilized hypochlorous acid (HOCl) and myeloperoxidase-
H$_2$O$_2$-Cl$^-$ to mimic the oxidation found in the cell. The oxidation of 3',5'-di-O-acetyl-2'-
deoxyguanosinse (AcGuo) by HOCl and H$_2$O$_2$-Cl$^-$ afforded six oxidation products
(Suzuki et al. 2003). Due to prior complications in retention times of the very polar
oxidation products using RP-HPLC, 2'-dG was acetylated to improve the separation. The
oxidation products included 3',5'-di-O-acetyl deoxyribonucleoside derivatives of
spiroiminodihydantoin (dSph), guanidinohydantoin-iminoallantoin (Gh-Ia), diamino-
oxazolone (Z), amino-imidazole (Iz), diimino-imidazole (Diz), and 8-Cl-Guo (Figure
2.4). The formation of AcdSph and AcdGh-AcdIa is proposed through chlorination of N-
7 followed by oxidative addition of H$_2$O to form 8-oxo-dGuo. It is then proposed that 5-
OH-8-oxo-AcdGuo is formed through either the addition of Cl$^-$ ion to C-4 to C-5 double
bond and subsequent attack by H$_2$O on C-5 or a two electron oxidation by HOCl forming
a cation which can then be hydrolyzed at C-5. It is also demonstrated that in neutral pH
the AcdSph is formed, whereas, in acidic pH AcdGh-AcdIa are formed.

Metals

In the one-electron oxidation of 7,8-dihydro-8-oxo-guanosine, the major products
were the equilibrating isomers of Guanidinohydantoin (Gh) and an Iminoallantoin (Ia)
nucleoside (Luo et al., 2001). This one-electron oxidation used iridium (IV) salts
Na$_2$IrCl$_6$ and Na$_2$IrBr$_6$ to model various one electron oxidants that may be present in the
cell. The proposed oxidation mechanism follows an initial formation of 5-hydroxy-8-
8-oxo-7,8-dihydroguanosine, which then undergoes hydration and decarboxylation to form
a mixture of Gh/Ia or Sp (Figure 2.5) (Luo et al., 2001). These intermediate and
subsequent products are also seen in the four-electron oxidation of guanosine by singlet oxygen, as seen in Figure 2.2.

In another study, with Mn-TMPyP/KHSO$_5$ (manganese (III)-bis(aqua)-meso-tetrakis(4- N-methylpyridiniumyl)-porphyrin) as an oxidizing agent, guanine in double-stranded DNA yielded imidazolone and parabanic acid derivatives (Vialas et al., 2000). 5,8-dihydroxy-7,8-dihydroguanine was characterized as an intermediate in this reaction (Figure 2.6).

Chworos et al. reported that the reaction of the same metal-oxo porphyrin (Mn-TMPyP/KHSO$_5$ from KHSO$_5$ + manganese (III)-bis(aqua)-meso-tetrakis(4-N-methylpyridiniumyl)-porphyrin) with 2'-deoxyguanosine affords a cation radical at either the C-8 position or at the C-5 position (Chworos et al. 2002). This oxidation of guanine by a metal-oxo porphyrin occurs through a 2-electron process. It is proposed that the C-8 cation is trapped by either a molecule of water (route a) or a molecule of KHSO$_5$ (route b) to form 8-oxo-7,8-dihydroguanosine or an oxidized form of 8-oxo-7,8-dihydroguanaine respectively (Figure 2.7). Alternatively, it is proposed that the C-5 cation is also trapped by either a molecule of water (route a) or a molecule of KHSO$_5$ (route b), to yield a product that is 10 mass units less than 2'-dG (G-10) and a product that is 12 mass units less than 2'-dG (G-12) respectively (Figure 2.8). These products undergo further oxidation at the C-8 position by either water or Mn$^{	ext{V}}$=O to afford guanidonhydantoin and a dehydro-guanidinoxydantoin, in that order (Figure 2.9).

Peroxynitrite

Peroxynitrite is formed by the in vivo diffusion-limited reaction between nitric oxide and superoxide (Huie and Padmaja, 1993). Peroxynitrite could be implicated in
DNA damage and therefore has a carcinogenic potential. Therefore, the following experiments have been conducted to attempt to mimic in vivo peroxynitrite reactions.

Reacting 2'-dG with peroxynitrite gave 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2'-deoxyguanosine (Douki et al., 1996) (Figure 2.10). The position of the hydroxyl and ONO groups was determined from the carbon chemical shifts. The C-4 resonance at δ = 145.8ppm versus the C-5 at δ = 135.5ppm made the C-4 position “more likely to carry the ONO group” because it is more electronegative than a hydroxyl group (Douki et al., 1996). However, this assignment was not rigorously confirmed.

In another experiment, oxidation of 8-oxoguanosine by peroxynitrite and KHSO₅/CoCl₂ led to the formation of spiroimidihydantoin, guaninidohydantoin, 4-hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid and others (Niles et al., 2004). The overall proposed mechanism is complex and involved multiple oxidations and rearrangements (Figure 2.11).
DMDO

Dimethyldioxirane (DMDO) is a three-membered cyclic peroxide that efficiently transfers oxygen and reacts selectively (Adam et al., 1989). The preparation method of DMDO reported in the literature yields a 0.1M solution (Adam et al., 1987). DMDO demonstrates efficient transfer of one atom of oxygen through a single concerted step (Edwards et al., 1979). This transfer of oxygen occurs through epoxidation of olefinic substrates (Adam et al., 1989, Murray et al., 1985, Edwards et al., 1979, and Baumstark et al., 1987). For the above reasons, DMDO is an excellent choice for use in a mild and general oxidation method for nucleosides and nucleotides.

In one report, DMDO selectively oxidized pyrimidine derivatives (Saladino et al., 1995 b). It was found that in the presence of either water or methanol, the epoxide formed by DMDO underwent nucleophilic ring opening to yield corresponding diol, methoxy-hydroxy, or hydroxyl pyrimidines (Figure 2.12). However, it is important to note that in this research, real DNA bases were not used.

In another report, Saladino et al. describe an efficient synthesis of 8-hydroxypurine derivatives through oxidation by DMDO (Saladino et al. 1995 a). Both caffeine and trityladenosine formed a reactive oxaziridine derivative to yield acceptable amounts of the corresponding 8-hydroxy compounds (Figure 2.13 and Figure 2.14).

Novel oxidation products were reported from oxidation of 2'-dG with DMDO (Davies et al., 2002). Addition of DMDO to 2'-deoxyguanosine and then heating to 90°C for 5 hrs afforded the product 4-aminocarbamoyl-5-hydroxyimidazole. The proposed mechanism involved epoxidation of the 4,5 carbon double bond by DMDO (Figure 2.15). This epoxide then underwent hydrolysis to form 4,5-dihydroxy-2'-deoxyguanosine. This
intermediate rearranged to give 4-aminocarbamoyl-5-hydroxyimidazole, however, the specifics of this mechanism step were not discussed in the paper.

My research determined that 2'-dG and 8-oxo-2'-dG followed an oxidation pathway similar to that proposed by Davies et al., in which DMDO forms a C4-C5 epoxide. However, as observed by Saladino and Bernini et al., and in contrast to Davies et al., the solvents water and methanol act as nucleophiles, opening the epoxide ring to yield methoxy and hydroxy substituted products.
Figure 2.1: Mechanism of formation of the main photo oxidation products of dGuo (1) and 3',5'-di-O-acetyl-2'-deoxyguanosine (1a) in neutral aqueous solution (Ravanat et al., 1995).

Figure 2.2: Oxidation pathways of Guanosine (Ye et al., 2003)
Figure 2.3: Possible mechanisms for the OH\(^{-}\) addition to, and one-electron oxidation of, dG (1) giving 8-oxodG (4), and further oxidation of 8-oxodG (4) ((Hofer et al., 2001).
Figure 2.4: Proposed mechanism for the reaction of AcdGuo with HOCl\(^\circ\) (Suzuki et al. 2003).

Figure 2.5: Proposed pathway for Ir\(^\text{IV}\) - mediated oxidation of 8-oxo-2'-deoxyguanosine (Luo et al., 2001).
Figure 2.6: Proposed mechanism of guanine oxidation within ds DNA by Mn-TMPyP/KHSO5 (Vialas et al., 2000).

\[ \text{dR} = \text{2-deoxyribose residue} \]

Figure 2.7: Proposed mechanism of guanine oxidation through a C-8 cation (Chworos et al. 2002).
dR = 2-deoxyribose residue

Figure 2.8: Proposed mechanism of guanine oxidation through a C-5 cation (Chworos et al. 2002).

dR = 2-deoxyribose residue

Figure 2.9: Proposed mechanism of further G-10 and G-12 C-8 oxidation (Chworos et al. 2002).

Figure 2.10: Structure of 4,5-dihydro-5-hydroxy-4-nitrosooxy-2'-deoxyguanosine (Douki et al., 1996).
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Figure 2.13: Proposed mechanism of Oxidation of caffeine with dioxirane (Saladino and Crestini et al., 1995).
Figure 2.14: Proposed mechanism of oxidation of adenosine with dioxirane (Saladino and Crestini et al., 1995).

Figure 2.15: Proposed mechanism of oxidation of rG and dG by DMDO (Davies et al., 2002)
Chapter 3

Materials and Methods

Chemicals

Oxone® (potassium peroxymonosulfate – H₃K₄O₁₈S₄), 2'-deoxyguanosine monohydrate, 8-hydroxy-2'-deoxyguanosine, sodium bicarbonate, sodium sulfate, HPLC grade acetone and HPLC grade methanol were purchased from Sigma-Aldrich (Milwaukee, Wisconsin).

Synthesis of DMDO

Dimethyldioxirane was synthesized by a slight modification of a procedure reported by Adam et al. (Adam and Chan et al., 1987). To perform this synthesis the following glassware were required: 2L 3-necked round bottom flask, solid addition funnel, condenser, distillation head, argon adapter, cold finger condenser, vacuum adapter, and round bottom flask for collection (see Appendix B for set-up diagram). To begin, sodium bicarbonate (36g) was added to the 2L flask, followed by a mixture 60mL of d.i. H₂O and 39mL reagent grade acetone, added through the neck with the condenser in order to keep the solid addition side dry. Stirring commenced. After the solid addition funnel was filled with 75g of Oxone, the system was purged with Argon. The cold traps were also filled and cooled to -78°C using dry ice and acetone. The Oxone was added slowly until complete (a significant amount of gas generation was observed). Due to the foaming at this stage, caution was used in the commencement of distillation under house vacuum. Upon completion of distillation (~20 minutes), a pink color developed in 2L.
round bottom flask and the vacuum was disconnected. Anhydrous Na₂SO₄ was added to the distillate until it was no longer cloudy. At this point, the solution was yellow. The solution of DMDO in acetone was then filtered through glass wool and stored at -80°C. The distillate is expected to be 0.1M of DMDO in acetone.

**Titration of DMDO**

DMDO was titrated to confirm the concentration. DMDO in acetone (1mL) was added to a solution of acetic acid-acetone (2mL; 3:2) and saturated aq. potassium iodide (2mL). A small piece of dry ice was added to de-aerate the solution and the mixture was left in the dark for 10 min. The sample was diluted with distilled water (5mL) and a 1mL aliquot was taken and titrated against an aqueous solution of sodium thiosulphate (1mM). The titration was stopped when a color change from dark brown to clear was observed. The titration was repeated 3 times to get the average volume of sodium thiosulphate used.

Reaction of DMDO with I₂: \( \text{DMDO} + 2 \text{I}^- + \text{H}_2\text{O} \rightarrow \text{I}_2 + \text{acetone} + 2\text{OH}^- \)

Titration Reaction: \( \text{I}_2 + 2 \text{Na}_2\text{S}_2\text{O}_3 \rightarrow 2 \text{NaI} + \text{Na}_2\text{S}_4\text{O}_6 \)

Sample Calculation:

\[
\text{[Na}_2\text{S}_2\text{O}_3] = 1\text{mM} \\
\text{[DMDO]} = \frac{\text{[I}_2]}{2} = \frac{\text{[Na}_2\text{S}_2\text{O}_3]}{2}
\]

Since the total volume of the sample equals 10mL,

\[
\text{[DMDO]} = \left( \frac{\text{[Na}_2\text{S}_2\text{O}_3] \times \text{Average Titration}}{\text{x 10}} \right) / 2.
\]

Specific Calculation:

\[
\text{[Na}_2\text{S}_2\text{O}_3] = \left( \frac{(0.0809\text{gm in 250mL})}{248.18} \right) \times 4 = 0.0013039\text{M}
\]

\[
\text{[DMDO]} = \left( \frac{\text{[Na}_2\text{S}_2\text{O}_3] \times \text{Average Titration}}{\text{x 9mL}} \right) / 2
\]

*We used 9mL because the 1mL had been taken out of the 5mL solution of DMDO, acetic acid-acetone, and potassium iodide before the 5mL of distilled water was added. There was 0.8mL of DMDO in the total 9mL solution.

\[
\text{[DMDO]} = \left( \frac{0.0013039\text{M}}{13.3\text{mL}} \times (9\text{mL}/0.8^*) \right) / 2
\]

\[
\text{[DMDO]} = 0.0975\text{M}
\]

The concentration of DMDO was calculated to be 0.097 mol/L.
Oxidation Procedure

The methods used in the oxidations of 2'-dG and 8-oxo-2'-dG were based upon the solubility of 2'-dG and 8-oxo-2'-dG and the characteristics of DMDO. The same procedure could be applied to either 2'-dG or 8-oxo-2'-dG. Although there was an initial question as to the possibility of DMDO oxidizing the NH groups, it was decided that because DMDO is selective for double bonds, it should not require initial protection of NH groups on 2'-dG or 8-oxo-2'-dG (Adam et al., 1989). Using an ice bath, a solution of 2'-dG (or 8-oxo-2'-dG) in 1:1 H2O and MeOH (1.25mg/mL) was treated with a solution of DMDO/acetone (0.1M) in ratios ranging from 1:1 to 5:1. The reaction mixture was stirred for 30 minutes at 0°C. Excess acetone and MeOH were evaporated under an Argon stream. The reaction mixture was stored at -80°C until analysis. DMDO was titrated prior to the experiment to ensure addition of the desired ratio. (See Tables 3.1 and 3.2 for specific reaction details for and work-up methods.)

For the majority of the reactions conducted, either 2'-dG or 8-oxo-2'-dG was dissolved in a mixture of water and methanol. However, it was later found that 2'-dG was soluble in water if a concentration of 0.9 mg/mL or lower was used. The effect of varying the ratios of 2'-dG or 8-oxo-2'-dG to DMDO on product profiles was investigated. Precautions need to be taken when working with DMDO. DMDO decomposes rapidly at ambient temperatures and is also volatile and therefore must be kept at -80°C. For this reason, the volumetric transfer of DMDO was difficult. To facilitate this, the pipette was pre-chilled in the -80°C freezer.
Analytical Instrumentation

In order to characterize these novel products, a number of analytical tools were utilized. These analytical tools included: Mass Spectrometry, proton NMR, HPLC (High Performance Liquid Chromatography), and UV detection.

Mass Spectrometry

Mass Spectrometry was the major analytical technique used. Mass Spectrometry enabled us to detect the molecular ions (positive ion mode) and major fragments of the oxidation products. Fragmentation patterns obtained by MS/MS were useful in determining the structures of the oxidation products. The Mass Spectrometry was performed on an LCQ Deca ion trap MS (Finnigan, San Jose, CA) with an electrospray ionization (ESI) source, used in positive mode. A mobile phase of ACN: H2O (1:1) 1%Acetic Acid was used at a flow rate 100 μL/min. Loop Injection with a 5-μL loop was used to introduce the entire mixture of products at one time.

Nuclear Magnetic Resonance Spectroscopy

The second tool employed in characterizing the novel products was Nuclear Magnetic Resonance (NMR) analysis. Proton NMR analysis was helpful in identifying the compounds through shifts and couplings of proton resonances. 1H NMR spectra were recorded on a Varian Inova 500 spectrometer at 500 MHz. The solvents consisted of DMSO-d6, D2O, or DMSO-d6 and D2O. D2O was used to identify exchangeable protons. Two-dimensional COSY (Correlation Spectroscopy) NMR was used to examine the connectivity between adjacent proton resonances.
High Pressure Liquid Chromatography

In order to attempt to characterize the individual products of the reaction mixture, HPLC was used. Preparative HPLC allowed separation of sufficient quantities products for spectroscopic analysis. A Varian Vista Series - 5000 Liquid Chromatograph was used with a Perkin-Elmer LS 85 C UV detector set at 253.7nm. Initially, a reverse phase C18 250 x 4.6mm phenomonex ultramex 5C18 column was used. On this reverse phase column, the compounds with the highest polarity elute first. Therefore, a high ratio of H2O to methanol was used to promote the more polar compounds to stay on the column. However, it was subsequently determined that the normal phase Alltech NH2 Econosphere 10μ, 250mm, 10mm column achieved better product separation. With the normal phase column, the elution order was reversed with the more polar compounds eluting later. Therefore, less water was used; the mobile phase varied from 10%-20% water and 80%-90% acetonitrile depending on the reaction mixture being separated. The flow varied from 4.0mL/min to 6.0mL/min.

After separation, UV profiles of the individual peaks collected were obtained using a Varian Cary 300 Bio UV-Visible spectrophotometer.
<table>
<thead>
<tr>
<th>Rxn</th>
<th>Date</th>
<th>Ratio of DMDO: 2′dG</th>
<th>Solvents</th>
<th>Time stirring on ice bath</th>
<th>Time stirring at room temp</th>
<th>Method of Drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9/4/03</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>1 hr</td>
<td>3hrs under Argon stream</td>
<td>Next day- Rotovap room temp bath</td>
</tr>
<tr>
<td>1c</td>
<td>9/16/03</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>30min</td>
<td>5hrs</td>
<td>Next day- blew Argon 2hrs/ left in water for HPLC Sep</td>
</tr>
<tr>
<td>3</td>
<td>11/10/03</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>30min</td>
<td>4hrs45min</td>
<td>Rotovap at 30C</td>
</tr>
<tr>
<td>4</td>
<td>12/1/03</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>1hr20min</td>
<td>None</td>
<td>Blow argon on for 5min/didn’t dry, quickly did D2O NMR</td>
</tr>
<tr>
<td>7</td>
<td>12/15/03</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>30min</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>12</td>
<td>1/31/04</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>30min / 3hrs Argon stream</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>17</td>
<td>3/17/04</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>30min / 3hrs Argon stream</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>18</td>
<td>3/17/04</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>blow Argon</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>19</td>
<td>4/5/04</td>
<td>3:1</td>
<td>MeOH/H2O</td>
<td>blow Argon</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>20</td>
<td>4/26/04</td>
<td>1:1 ≈ H2O</td>
<td></td>
<td>45min</td>
<td>1hr15min/under Argon stream for 15min</td>
<td>Speed Vac (2days)</td>
</tr>
<tr>
<td>21</td>
<td>4/26/04</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>1hr15min</td>
<td>1hr under Argon stream</td>
<td>Speed Vac (2days)</td>
</tr>
<tr>
<td>22</td>
<td>5/5/04</td>
<td>5:1</td>
<td>H2O</td>
<td>30min</td>
<td>1hr then 3hrs under Argon stream</td>
<td>Speed Vac (1day)</td>
</tr>
<tr>
<td>24</td>
<td>6/2/04</td>
<td>4:1</td>
<td>MeOH/H2O</td>
<td>40min</td>
<td>1hr under Argon stream</td>
<td>Rotovap at 30C</td>
</tr>
<tr>
<td>26</td>
<td>6/16/04</td>
<td>4:1</td>
<td>PhosBuff pH6</td>
<td>40min</td>
<td>30min under Argon stream</td>
<td>Speed Vac (1day)</td>
</tr>
<tr>
<td>27</td>
<td>6/16/04</td>
<td>4:1</td>
<td>H2O = pH 5</td>
<td>40min</td>
<td>30min under Argon stream</td>
<td>Speed Vac (1day)</td>
</tr>
<tr>
<td>28</td>
<td>6/16/04</td>
<td>4:1</td>
<td>PhosBuff pH8</td>
<td>40min</td>
<td>30min under Argon stream</td>
<td>Speed Vac (1day)</td>
</tr>
<tr>
<td>29</td>
<td>7/7/04</td>
<td>4:1</td>
<td>H2O = pH5</td>
<td>30min</td>
<td>30min under Argon stream</td>
<td>Speed Vac</td>
</tr>
</tbody>
</table>
Table 3.2: 8-oxo-2'-dG Reaction Method Details

<table>
<thead>
<tr>
<th>Rxn</th>
<th>Date</th>
<th>Ratio of DMDO to 8-oxo-2'-dG</th>
<th>Solvents</th>
<th>Time stirring on ice bath</th>
<th>Time stirring at room temp</th>
<th>Method of Drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12/4/03</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>45min</td>
<td>None</td>
<td>Blow Argon 4hrs on ice bath/then sep with HPLC</td>
</tr>
<tr>
<td>8</td>
<td>12/15/03</td>
<td>1:1</td>
<td>CD3OD/D2O</td>
<td>30min/5 min under Argon stream</td>
<td>None</td>
<td>5min blow Argon on ice bath, then put in NMR tube</td>
</tr>
<tr>
<td>9</td>
<td>1/15/04</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>30min/5 min under Argon stream</td>
<td>None</td>
<td>5min blow Argon on ice bath, later lyophilizer</td>
</tr>
<tr>
<td>10</td>
<td>1/15/04</td>
<td>1:1</td>
<td>CD3OD/D2O</td>
<td>30min/5 min under Argon stream</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>1/31/04</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>30min</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>2/24/04</td>
<td>4:1</td>
<td>MeOH/H2O</td>
<td>30min/30min under Argon stream</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>15</td>
<td>2/24/04</td>
<td>4:1</td>
<td>CD3OD/D2O</td>
<td>30min/30min under Argon stream</td>
<td>None</td>
<td>Lyophilizer</td>
</tr>
<tr>
<td>23</td>
<td>5/18/04</td>
<td>1:1</td>
<td>MeOH/H2O</td>
<td>45min/1hr 20min under Argon stream</td>
<td>None</td>
<td>Speed vac 3hrs</td>
</tr>
</tbody>
</table>
Chapter 4

Results

The ratio of DMDO to 2'-dG or 8-oxo-2'-dG was varied in order to observe the formation of various oxidation products and to help determine the mechanism of oxidation. It was expected that DMDO oxidation of 2'-dG would follow the same pathway as the oxidation of 2'-dA, giving the C8-N7 epoxide with rearrangement to 8-oxo-2'-dG (Saladino et al., 1995a). With excess DMDO, it was also hypothesized that further oxidation products reported in the literature could be formed. These consisted of 8-oxo-2'-dG, 4-OH-8-oxo-2'-dG, 5-OH-8-oxo-2'-dG, and spiroiminodihydantoin, guanidinohydantoin-iminoallantoin, diamino-oxazolone, amino-imidazolone, and diiminoimidazole derivatives (Ravanat et al., 1995; Hofer, 2001; and Suzuki et al, 2003). It was conjectured that the same oxidation products could be formed in the oxidation of 8-oxo-2'-dG by DMDO. Mass Spectrometry was used to screen crude reaction mixtures, followed by NMR. Selected reactions were fractionated by HPLC. The results of the oxidations of 2'-dG and 8-oxo-2'-dG are reported below.

Oxidation of 2'-dG

DMDO 1:2 2'-dG

In the reaction of DMDO/2'-dG in a 1:2 ratio, it was expected that due to the limited amount of oxidizing agent, up to 50% of unreacted 2'-dG would remain, and accordingly, a limited amount of oxidation products would be formed. Figure 4.1 shows
the mass spectrum of a reaction in which a 1:2 ratio of DMDO to 2'-dG was used. In this figure, the relative abundance is on the y-axis, the mass-to-charge ratio is on the x-axis, and the structures corresponding to the molecular ions are shown. The major ions at even m/z, in positive ion mode correspond to odd ion masses, which indicate that these ions have an odd number of nitrogen atoms (McLafferty, 1963). The molecular ion at m/z 268 corresponds to unreacted 2'-dG; the molecular ion at m/z 284 represents the addition of one atom of oxygen to 268, which is most likely to be 8-oxo-2'-dG; and m/z 302, which is the addition of 34 mass units, presumed to represent 2 hydroxyl groups, to give 4,5-dihydro-4,5-dihydroxy-2'-dG (4,5-diol-2'-dG). The molecular ion at m/z 316 represents the addition of 48 mass units and is assigned the structure 4,5-dihydro-4-hydroxy-5-methoxy-2'-dG.

DMDO 1:1 2'-dG

Figure 4.2 shows the mass spectrum of a reaction in which DMDO was used in a 1:1 ratio to 2'-dG. In this crude reaction mixture, the four ions at m/z 268, 284, 302, and 316 as described in the mass spectrum of the previous reaction, are also seen in this mass spectrum. However, a new product appears at m/z 274, corresponding to the molecular ion of Guanidinohydantoin (Gh).

DMDO 2:1 2'-dG

Figure 4.3 shows the full mass spectrum of the reaction in which a 2:1 ratio of DMDO to 2'-dG was used. The absence of a peak at 268 (2'-dG) indicates that 2'-dG was completely consumed. The ions in the mass spectrum represent Gh (274 m/z) and 4,5-dihydro-4,5-dihydroxy-2'-dG (302 m/z).
Reaction 21

As noted in Chapter 3, Reaction 21 was carried out by reacting DMDO in a 2:1 ratio with 2'-dG dissolved in MeOH/H₂O 1:1 by volume. The mass spectrum of the crude reaction mixture yielded ions that could be attributed to expected oxidation products (Figure 4.4). The molecular ion at m/z 302 corresponds to 4,5-diol-2'-dG. The ion at m/z 274 represents Gh. The ions of the corresponding bases of 4,5-diol-2'-dG and Gh without the sugars are also present at m/z 186 and m/z 158, respectively. The ions 286 and 314 were not consistently in product mass spectra and are currently attributed to background noise. The molecular ions at m/z 324 and m/z 208 are the sodium adducts of the ions at m/z 302 and 186, respectively. It is important to note that in this 2:1 reaction ratio, there is no unreacted 2'-dG (268 m/z) or 8-oxo-2'-dG (284 m/z).

To further confirm the structure of these oxidation products, tandem mass spectrometry was used to select a particular ion and collisionally activate it in order to determine the fragmentation pattern. Figure 4.5 is an MS/MS of molecular ion 302 which corresponds to 4,5-dihydro-4,5-dihydroxy-2'-dG. The fragments are consistent with the assigned structure: m/z 284 is the loss of water (MH-18); m/z 186, loss of sugar (MH-116); m/z 158, loss of sugar and loss of carbon monoxide (MH-144); m/z 141, loss of sugar, CO, and OH (MH-161). The MS/MS of the ion at m/z 274 demonstrates a major loss of CO and CN₂H₃ (MH-85) at m/z 189, a minor loss of the deoxyribose (MH-116) at m/z 158, and the loss of deoxyribose and OH (MH-133) (Figure 4.6).

Comparison of the MS/MS from the ions at m/z 302 and 274 shows that m/z 274 does not arise from loss of 28 (CO) from 302 because m/z 302 and 274 follow different fragmentation patterns and m/z 274 is not seen in the MS/MS of m/z 302.
Nuclear Magnetic Resonance (NMR) spectroscopy contributed to the structural
categorization of these compounds. Mass Spectrometry identified the molecular ions,
but the NMR data helped to further confirm the structure.

Figure 4.7 shows the NMR spectrum of crude Reaction 21 in DMSO-d$_6$, in which,
4,5-Dihydro-4,5-dihydroxy-G (4,5-diol-G) is the major product. The integration values
consistent with 4,5-diol-G and $\alpha$ and $\beta$ anomers of deoxyribose (Figure 4.9) clearly
present in the Reaction 21 NMR, confirm that Reaction 21, as isolated, contained
predominantly deglycosylated products (Figure 4.7). Comparison of the NMR spectrum
in Figure 4.7 with that of the starting material (2'-dG) (Figure 4.8) is informative
regarding the compounds in Reaction 21. The presence of peaks assigned to H$_3$ in Figure
4.8 proves that oxidation does not occur at the C-8 position. In Figure 4.10, D$_2$O was
added to exchange all of the non-carbon protons, confirming the identities of the C-8
proton and ring protons of 2-deoxyribose.

The NMR Spectrum of crude Reaction 21 was deconvoluted in part
through matching signals with 2-deoxyribose (Figure 4.9) and 2'-dG (Figure 4.8)
standards. The identification of the signals of the $\beta$ 2-deoxyribose was confirmed in the
2-D COSY NMR spectrum of Reaction 29 (Full Spectrum Figure 4.11) (Figure 4.12). In
this spectrum, a cross peak is present between the H-1' $\beta$ and the H-2'/H-2" $\beta$ of the
deoxyribose, indicating that they occupy adjacent positions. Accordingly, cross peaks are
present between the H-2'/H-2" $\beta$ and the H-3' $\beta$ and between the H-3' $\beta$ and the H-4' $\beta$.
Due to the multiple products present in this spectrum and the large water peak, the cross
peak that would normally be present between the H-4' $\beta$ and H-5'/H-5'' $\beta$ is obscured. In
addition, the complex COSY spectrum allowed for only partial confirmation of the
identification of the signals of the α 2-deoxyribose (Figure 4.13). Although, the cross peaks are present between the H-1′ α and the H-2′/H-2″ α and between the H-2′/H-2″ α and the H-3′ α, the cross peaks normally present between the H-3′ α and the H-4′ α and between the H-4′ α and H-5′/H-5″ α cannot be detected. In the COSY spectrum, a cross peak is present between the 9-NH and H-8, confirming that the protons are adjacent and there is a loss of sugar (Figure 4.14). A number of cross peaks in the COSY spectrum suggest the cis conformation of the 4,5-diol-2′-dG structure. In addition to the COSY interactions, cross peaks appear to contain both COSY and NOESY information since cross peaks between the 4-OH and 5-OH are likely to be the result of through-space interactions. However, due to observed line-broadening, multiple products, and overlapping absorptions in the 1H-NMR spectrum of the crude reaction mixture, it was necessary to separate the products for further characterization.

**Separation of oxidation products**

**Rxn 21 Peak 1**

The mixture of oxidation products of Reaction 21 were separated by HPLC (Figure 4.15). On an amino column, using 15% water and 85% acetonitrile with the UV detector set at 253.7nm, three peaks were isolated with retention times of 12, 16 and 21 min.

The mass spectrum of Reaction 21, Peak 1, (RT = 12 min) is presented in Figure 4.16. The base peak at m/z 186 corresponds to the deglycosylated diol product 4,5-dihydro-4,5-dihydroxy-guanine (4,5-diol-G). Figure 4.17 shows the MS/MS fragmentation pattern of 4,5-diol-G. As in the case of 4,5-diol-2′-dG, the molecule loses •OH (MH-17) to give m/z 169; CO (MH-28) to give m/z 158; and COOH (MH-45) to
give m/z 141. The $\lambda$ max of (4,5-diol-G) in 15% Water and 85% Acetonitrile was 231.83nm (Appendix C Figure C.3).

The NMR spectrum of Reaction 21 Peak 1 in DMSO-$d_6$ shows peaks we propose to be characteristic of 4,5-diol-G (Figure 4.18). Compared to the NMR spectrum of 2'-dG (2'-dG), the C-8 proton of 4,5-diol-G does not shift very much from the C-8 proton of 2'-dG (Figure 4.8). The exocyclic amino group (NH$_2$) appears as a pair of very broad singlets overlapping H-8 at approximately 8.0ppm. It is important to note that the large water peak could be contributing to the broadening of these NH peaks. The N-9 appears at 8.8ppm. The 4-OH and 5-OH protons appear as relatively sharp singlets at 7.19 and 7.29ppm. These proton peaks (N-9, 4-OH, and 5-OH) are not present in the NMR spectrum of 2'-dG indicating the change in the parent compound. The peak at 8.3ppm integrated to widely different values in different samples, hence, it was determined that this peak was a contamination from the HPLC separation.

**Reaction 21 Peak 2**

The second peak collected from HPLC had a retention time of 16 min. The mass spectrum of Reaction 21, Peak 2, shows both 4,5-diol-2'-dG and 4,5-diol-G to be to be present due to the major peak at m/z 302 and the minor peak at m/z 186, respectively (Figure 4.19). The $\lambda$ max of Reaction 21 Peak 2 in 15% Water and 85% Acetonitrile was found to be 231.17nm (Appendix C Figure C.4). In the NMR spectrum of Reaction 21, Peak 2, 4,5-diol-G appears to be the only observable product in the weak spectrum (Figure 4.20).

**Reaction 21 Peak 3**
The third peak collected from HPLC had a retention time of 21 min. As in Reaction 21, Peak 2, the mass spectrum of Reaction 21, Peak 3, shows that 4,5-diol-2'-dG and 4,5-diol-G both seem to be present. However, other ions of unknown compounds are also present (Figure 4.21). The λ max of Reaction 21, Peak 3, in 15% water and 85% acetonitrile was not as pronounced as in the other peaks, however it seemed to be around 231nm as well (Appendix C Figure C.5). The NMR Spectrum of Reaction 21 Peak 3 appears to indicate that the largest component of this fraction is column bleed represented by the large peak at 8.3ppm (Figure 4.22).

**4,5-Diol compound at varied ratios**

At varying ratios of DMDO to 2'-dG, one product was consistently formed. This product is 4,5-dihydro-4,5-dihydroxy-2'-dG (4,5-diol-2'-dG). This product was also seen after deglycosylation (4,5-dihydro-4,5-dihydroxy-Guanine (4,5-diol-G). The structure of the product, 4,5-diol-G, was confirmed based on Mass Spectrometry and NMR.

As shown in Table 4.1, 4,5-diol-G and 4,5-diol-2'-dG are formed in reactions that have a 2:1 ratio or a 4:1 ratio of DMDO to 2'-dG. Following Table 4.1, Mass Spectra and NMR Spectra are given in order of reaction number (Figure 4.23 – Figure 4.28). (See Figure 4.4 for the Mass Spectrum of Reaction 21 crude and Figure 4.7 for the NMR Spectrum of Reaction 21 crude in DMSO-d₆.)
8-oxo-2'-dG Results

DMDO 1:1 8-oxo-2'-dG

Figure 4.29 is the mass spectrum containing the molecular ions of the crude reaction mixture of 8-oxo-2-dG reacted in a 1:1 ratio with DMDO. The molecular ions at m/z 284, 318, and 332 are representative of 8-oxo-2'-dG, 4,5-dihydro-4,5-dihydroxy-8-oxo-2'-dG (4,5-diol-8-oxo-2'-dG), and 4,5-dihydro-4-methoxy-5-hydroxy-8-oxo-2'-dG, in that order. In this spectrum, it is important to note that the ion at m/z 274 is not observed, indicating that guanidinohydantoin (Gh) is not formed at the 1:1 ratio.

DMDO 2:1 8-oxo-2'-dG

Figure 4.30 is the mass spectrum of the reaction with a 2:1 ratio of DMDO to 8-oxo-2'-dG. This mass spectrum contains the molecular ions at m/z 318 and 335, which correspond to 4,5-diol-8-oxo-2'-dG and 4,5-dihydro-4-methoxy-5-hydroxy-8-oxo-2'-dG, respectively. However, differing from the 1:1 ratio reaction, the molecular ion of the starting material (284 m/z) is not present in this spectrum, but the molecular ion of Gh (274 m/z) is present. This is indicative of the excess oxidizing agent consuming all of the starting material.

MS/MS of 274

Figure 4.31 is the MS/MS of the 274 ion, which is the molecular ion that corresponds to Gh. In this spectrum, the fragmentation pattern for m/z 274 signifies the loss of guanidinyl and carbon monoxide (MH-86) at m/z 188. The fragment ion at m/z 173 indicates the loss of guanidinyl and cyanate (MH-101) from m/z 274. The subsequent loss of carbon monoxide gives a daughter fragment at m/z 145. The ion at
m/z 117 corresponds to the intact deoxyribose indicating it was not oxidized during the reaction.

**MS/MS of 318**

Figure 4.32 is a MS/MS of the molecular ion at m/z 318 (4,5-diol-8-oxo-2'-dG). The fragment at m/z 300 indicates the loss of H₂O (MH-18) from the molecule. The fragment at m/z 202 represents the loss of the deoxyribose (MH-116) sugar and the fragment ion at m/z 184 corresponds to a subsequent loss of H₂O (MH-134).

**MS/MS of 332 and 335**

To further investigate the methoxy-hydroxy compound, we conducted two experiments simultaneously. One reaction was carried out in a solution of methanol and water, whereas, the other reaction was carried out in deuterated methanol and water. This allowed determination of whether deuterated methanol would add to 8-oxo-2'-dG. If it did, the fragmenting patterns in the mass spectra would allow further validation of the proposed structure for 4,5-dihydro-4-methoxy-5-hydroxy-8-oxo-2'-dG (methoxy-hydroxy-8-oxo-2'-dG). Figure 4.33 presents two mass spectra comparing the MS/MS of 332 (protio) methoxy-hydroxy (top) and 335 (D) methoxy-d₁-hydroxy (bottom). The fragmentation pattern in the spectra are very similar with the presence of a fragment ion at m/z 300 correlating to the loss of methanol (-32) or the loss of deuterated methanol (-35). Both spectra also show the loss of water (-18) from the parent molecule, at m/z 311 and m/z 314, respectively. The loss of sugar observed in both spectra indicates that the deoxyribose stayed intact during the oxidation reaction. However, further confirmation that the methanol added to the base and not the sugar is due to the fact that even after the
deoxyribose is lost from the base, the ion at m/z 184 correlates to the subsequent loss of methanol and deuterated methanol from the base.

**NMR evidence for the Methoxy-hydroxy product**

Figure 4.34 is a spectrum of the 4,5-dihydro-4-methoxy-5-hydroxy-8-oxo-2’-dG (methoxy-hydroxy-8-oxo-2’-dG) molecule after RHPLC clean-up. In this spectrum, it can be seen that all of the deoxyribose peaks are present. The most important evidence gained from this spectrum is seen in the expansion of the characteristic methoxy peak at 3.7 ppm. The doubling of this peak is a clear indication that this product exists as two diastereomers. Due to the opening of the epoxide by methanol, it is possible to form 2 pairs of diastereomers due to the chiral centers at C-4 and C-5. However, the splitting of this peak indicates that only 2 diastereomers were formed. The N-H and O-H proton peaks were identified through proton exchange with D$_2$O (Figure 4.35). The O-H proton peak at 6.5ppm in Figure 4.29 is not seen in the NMR of the parent compound (Figure 4.36).
Chapter 4 Figures

DMDO 1:2 2'dG

Figure 4.1: Mass Spectrum of DMDO 1:2 2'-dG (Reaction 16)

DMDO 1:1 2'-dG

Mass Spec

Figure 4.2: Mass Spectrum of DMDO 1:1 2'-dG (Reaction 17)
DMDO 2:1 2'-dG

- Mass Spec
- 274
- 302

Figure 4.3: Mass Spectrum of DMDO 2:1 2'-dG (Reaction 3)

Figure 4.4: Mass Spectrum of crude reaction 21
Figure 4.5: MS/MS of Molecular Ion 302 (Reaction 3)

Figure 4.6: MS/MS of the molecular ion 274 (Reaction 3)
Figure 4.7: NMR Spectrum of Reaction 21 Crude in DMSO-d$_6$
Figure 4.8: NMR Spectrum of 2'-dG in DMSO-d₆
Figure 4.9: NMR Spectrum of 2-dexoyribose in DMSO-d₆ and D₂O

Figure 4.10: NMR Spectrum of Reaction 21 Crude in DMSO-d₆ and D₂O
Figure 4.11: COSY NMR Spectrum of crude reaction 29 in DMSO-$d_6$
Figure 4.12: Expanded COSY of crude reaction 29 in DMSO-d$_6$ (β 2-deoxyribose)
Figure 4.13: Expanded COSY of crude reaction 29 in DMSO-d$_6$ (α 2-deoxyribose)
Figure 4.14: Expanded COSY NMR Spectrum of crude reaction 29 in DMSO-d$_6$
Figure 4.15: HPLC profile of crude Reaction 21
Figure 4.16: Mass Spectrum Rxn 21 Peak 1

Figure 4.17: Mass Spectrum Rxn 21 Peak 1 Ms/MS of 186 (4,5-diol-G)
Figure 4.18: NMR Spectrum of Reaction 21 Peak 1 in DMSO-d$_6$

Figure 4.19: Mass Spectrum of Reaction 21 Peak 2
Figure 4.20: NMR Spectrum of Reaction 21 Peak 2 in DMSO-d6

Figure 4.21: Mass Spectrum of Reaction 21 Peak 3
Figure 4.22: NMR Spectrum of Reaction 21 Peak 3 in DMSO-d6

<table>
<thead>
<tr>
<th>Crude Reaction</th>
<th>HPLC Peak</th>
<th>Date of Reaction</th>
<th>Ratio of DMDO to 2'-dG</th>
<th>Conditions</th>
<th>186 Present in Mass Spec</th>
<th>UV</th>
<th>Peaks in NMR corresponding to 4,5-diol-G</th>
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<td>11/10/03</td>
<td>2:1</td>
<td>MeOH/H2O</td>
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<td>pk1-232-277</td>
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<td>4/26/04</td>
<td>2:1</td>
<td>MeOH/H2O</td>
<td>Yes</td>
<td>232</td>
<td>Yes</td>
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<tr>
<td></td>
<td>21 pk2</td>
<td></td>
<td></td>
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<td></td>
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<td>24</td>
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<td>232</td>
<td>Yes</td>
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</tbody>
</table>

Table 4.1: Table of 2'-dG Reactions and Results
Figure 4.23: Mass Spectrum of Reaction 3 pk1-2

Figure 4.24: NMR Spectrum crude reaction 3 peak 1-2 in DMSO-d₆
Figure 4.25: Mass Spectrum of Reaction 24 Crude

Figure 4.26: NMR Spectrum Reaction 24 Crude in DMSO-d$_6$
Figure 4.27: Mass Spectrum of Reaction 29 Crude

Figure 4.28: NMR Spectrum of Reaction 29 Crude in DMSO-\textit{d}_6
Figure 4.29: Mass Spectrum of DMDO 1:1 8-oxo-2'-dG
DMDO 2:1 8-oxo-2'-dG
Mass Spec Results

- 274 (major)
- 318 (minor)
- 332 (minor)

Figure 4.30: Mass Spectrum of DMDO 2:1 8-oxo-2'-dG
Figure 4.31: MS/MS of Molecular Ion 274 (Reaction 14)

Ms/MS 318

Formula Weight = 317.256
Molecular Formula = C_{20}H_{27}N_{11}O_{8}
4,5-dihydroxy-dihydro-8-oxo-2-deoxyguanosine

Figure 4.32: MS/MS of Molecular Ion 318 (reaction 8)
Figure 4.33: MS/MS of Molecular Ions 332 and 335 (reaction 14 and 15)

Figure 4.34: NMR spectrum of Methoxy-hydroxy in DMSO-d$_6$
Figure 4.35: NMR spectrum of Methoxy-hydroxy in DMSO-d$_6$ and D$_2$O

Figure 4.36: NMR Spectrum of 8-oxo-2'-dG in DMSO-d$_6$
Chapter 5
Discussion

In this research, the products and thus the mechanism for oxidation of both 2\textsuperscript{'}-deoxyguanosine and 8-oxo-deoxyguanosine by DMDO was found to be very similar (Figure 5.1).

Figure 5.1: Oxidation pathway of 2\textsuperscript{'}-dG and 8-oxo-2\textsuperscript{'}-dG by DMDO
2'-dG Oxidation

The 2'-dG oxidation products reported in the literature include: 8-oxo-2'-dG, 4-OH-8-oxo-2'-dG, 5-OH-8-oxo-2'-dG, and spiroiminodihydantoin, guanidinohydantoin-iminoallantoin, diamino-oxazolone, amino-imidazolone, and diimino-imidazole derivatives (Ravanat et al., 1995; Hofer, 2001; and Suzuki et al, 2003). However, the mechanism of oxidation and the oxidation products of 2'-dG formed from oxidation by DMDO are distinct from those arising from radical chemistry. The overall oxidation pathway and products of 2'-deoxyguanosine found in the present research are shown in Figure 5.2. Out of the products reported in the prior section, 4,5-dihydro-4,5-dihydroxy-2'-deoxyguanosine (4,5-diol-2'-dG) was the major product in the reaction mixture. For this compound, the first step in the oxidation pathway is the formation of an epoxide at the C-4 to C-5 carbon-carbon double bond (Figure 5.2). This epoxide is subsequently opened through hydrolysis, and thus forms the 4,5-diol-2'-dG product. Although 4,5-diol-2'-dG was found to be the major characterized product in the presence of methanol, the epoxide intermediate can also undergo methanolation to yield 4,5-dihydroxy-4-methoxy-5-hydroxy-2'-dG. Alternatively, in the formation of the other minor oxidation products of 2'-dG, the N-7 to C-8 double bond is initially oxidized, thus affording 8-oxo-2'-dG (Figure 5.3). This oxidation is then followed by an epoxidation of the C-4 to C-5 double bond. This intermediate can undergo either hydrolysis or methanolation. Methanolation of the intermediate yields 4,5-dihydro-5-hydroxy-4-methoxy-8-oxo-2'-dG. Alternatively, hydrolysis to the intermediate gives 4,5-dihydro-4,5-dihydroxy-8-oxo-2'-dG (4,5-diol-8-oxo-2'-dG). Subsequent hydrolysis and loss of carbon dioxide from 4,5-diol-8-oxo-2'-dG affords guanidinohydantoin.
Figure 5.2: Mechanism of DMDO Oxidation of 2'dG to 4,5-dihydro-4,5-dihydroxy-2'-dG

Figure 5.3: Mechanism of DMDO Oxidation of 2'dG to Guanidinohydantoin and 4,5-dihydro-5-hydroxy-4-methoxy-8-oxo-2'-dG

Although guanidinohydantoin, 4-hydroxy-5-methoxy-2'-dG, 8-oxo-2'-dG, 4,5-diol-8-oxo-2'-dG, and 4-methoxy-5-hydroxy-8-oxo-2'-dG are observed in the mass spectra of the 2'-dG reaction mixture, the major product consistently observed and subsequently characterized was 4,5-diol-2'-dG. This indicates that DMDO preferentially
oxidizes the C-4 to C-5 double bond over the C-8 to N-7 double bond of guanine. Observation of 4,5-diol-2'-dG as the major oxidation product also indicates that it is not subject to further oxidation at the C-8 and N-7 double bond. One possible explanation is that if the C-4 and C-5 bond is saturated, it decreases the reactivity of the C-8 and N-7 double bond.

Furthermore, the stability of 4,5-diol-2'-dG was questioned when it was found that the major peak collected after HPLC separation was the depurination product, 4,5-dihydro-4,5-dihydroxy-guanine. The difference in the mass spectra between crude reaction 21 with a major ion at m/z 302 (Figure 4.4) versus reaction 21 peak 1 with a base peak at m/z 186 (Figure 4.16) further confirms depurination during chromatographic purification. Thermal depurination follows a similar mechanism to acid catalyzed depurination (Gates et al., 2004). The modified guanine moiety has a reduced stability due to weakening of the glycosidic bond (Figure 5.4).

Figure 5.4: Depurination of 4,5-diol-2'dG

8-oxo-2’-dG Oxidation

Similarly to 2'-dG, 8-oxo-2’-dG undergoes oxidation by DMDO through epoxide formation at the C-4 to C-5 carbon-carbon double bond followed by solvolysis.
Hydrolysis of the epoxide intermediate yields the corresponding 4,5-dihydro-4,5-dihydroxy-8-oxo-2'-deoxyguanosine (Figure 5.6). Subsequent hydrolysis and loss of carbon dioxide affords the product guanidinohydantoin. Correspondingly, methanolysis 4,5-dihydro-5-hydroxy-4-methoxy-8-oxo-2'-deoxyguanosine (Figure 5.5).

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

**Figure 5.5: Oxidation and Hydrolysis/Methanolysis of 8-oxo-2'-dG**

**Alternative Proposed Mechanism**

Through examination of the results, it was found that hydrolysis is favored over methanolysis in both 2'-dG and 8-oxo-2'-dG. However, the difference is subtler in 8-oxo-2'-dG than in 2'-dG, as confirmed by the fact that the 2'-dG methoxy product was not able to be separated or consistently observed, whereas, the 8-oxo-2'-dG methoxy product was easily separated from the product mixture. This observation suggests that although both 2'-dG and 8-oxo-2'-dG both form an epoxide at the C-4 to C-5 double bond, the rate of epoxide ring opening and the ratio of product formation are different due to the differences in epoxide stability and solvolysis.
One possible explanation for the difference in product formation could be due to the fact that methanol is usually a poor choice for SN2 reactions. The epoxide with longer half-life, will allow both water and methanol time to initiate nucleophilic attack. 8-oxo-2'-dG affords a more stable epoxide than 2'-dG due to the electron-withdrawing effect of the carbonyl at the C-8 position, which impedes opening to form a carbonium ion. Conversely, the epoxide afforded from the 2'-dG oxidation is less stable and therefore, only water has a chance to react.

Another possible explanation for the difference in formation of the hydroxy-methoxy and dihydroxy products could be due to the immediate rearrangement of the 8-oxo-2'-dG epoxide to form 5-hydroxy-8-oxo-2'-dG (an intermediate which is stable enough to allow both water and methanol time to initiate nucleophilic attack). The proton on N-7 of 8-oxo-2'-dG allows this rearrangement to occur, whereas 2'-dG does not have an N-7 proton and therefore will not rearrange to 5-hydroxy-2'-dG. This type of rearrangement was also reported by Sheu and Foote, 1995.

Possible explanation for -OCH₃ substitution at C-4

At this time, there is insufficient data to determine the location of the methoxy group. However, it is proposed that methanol attacks at the C-4 carbon rather than the C-5 carbon due to the differences in stability of the carbonium ions. The C-5 carbonium ion is not as stable as the C-4 carbonium ion because it is adjacent to a carbonyl group, which impedes it from having a positive charge. Whereas, the methanol is more likely to attack at the C-4 position because the C-4 carbon is attached to two nitrogen atoms, which help to stabilize the positive charge, thus allowing methanol to initiate nucleophilic attack at the carbonium ion at C-4.
Conclusion

It is important to be able to identify the oxidation products of 2'-deoxyguanosine and 8-oxo-2'-deoxyguanosine. We report here, a straightforward preparative synthesis of these products through oxidation by DMDO. Future work will require that the reaction method be scaled up and an efficient method of separation of reaction products be found.

This research concludes that DMDO, which has an oxidation mechanism analogous to the mixed-function oxidases, generates products distinct from those arising from radical chemistry. These, new and interesting, DNA lesions, which were not previously reported or characterized, are now available for more informative studies of molecular mutagenesis and DNA repair.
References


Grollman AP and Moriya M. Mutagenesis by 8-oxoguanine: An Enemy Within. Trends Genet. 9 1993; 246-249.


Vialas C, Claparols C, Pratviel G, and Meunier B. Guanine Oxidation in Double-Stranded DNA by Mn-TMPyP/KHSO3: 5,8-Dihydroxy-7,8-dihydroguanine Residue as a Key Precursor of Imidazolone and Parabanic Acid Derivatives. J. Am. Chem. Soc. 2000; 122(10); 2157-2167.


Appendix A

Synthesis of DMDO

Methods:
1. Add 36g of NaHCO₃ to 2L flask
2. Then add 60mL d.i. H₂O, to 39ml reagent grade acetone. (Add this all through neck with condenser. Keep solid side dry.)
3. Stir
4. Fill solid addition funnel with 75g oxone
5. Purge with Argon
6. Fill cold traps and let cool to -78°C
7. Slowly add oxone, when addition is complete (observe lots of gas generation) replace argon gas adapter with stopper
8. Then distill using house vacuum.
9. Care is needed here as reaction foams at this stage
10. When distillation is complete, (observed pink color developed in 2L RB) disconnect vacuum.
11. Add anhydrous Na₂SO₄ to distillate until it is no longer cloudy. It should be yellow here.
12. Filter through glass wool, label, and keep it in -80°C degree freezer.

(2hrs. Reaction time 30min)

Materials Needed: 2Lvb, solid addition flask, condenser, distillation head, argon adapter, cold finger condenser, vacuum adapter, and vb for collection

Appendix B

UV Spectra

Figure C.1: UV spectrum of 2'-dG in 10% Water and 90% Acetonitrile

Figure C.2: UV spectrum of 8-oxo-2'-dG
Figure C.3: UV Spectrum of Rxn 21 Pk 1 in 15% Water and 85% Acetonitrile

Figure C.4: UV Spectrum of Rxn 21 Pk 2 in 15% Water and 85% Acetonitrile
Figure C.5: UV Spectrum of Rxn 21 Pk 3 in 15% Water and 85% Acetonitrile

Figure C.6: UV Spectrum of Rxn 29 Crude in 100% Water
Figure C.7: UV Spectrum of Rxn 29 Pk 1 in 20% Water and 80% Acetonitrile

Figure C.8: UV Spectrum of Rxn 29 Pk 2 in 20% Water and 80% Acetonitrile
Figure C.9: UV Spectrum of Rxn 29 Pk 3 in 20% Water and 80% Acetonitrile