Photochemical generation of the C5'-uridinyl and pseudouridinyl radical for the study of oxidative damage in RNA

Raziya Shaik

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A Dissertation

entitled

Photochemical Generation of the C5'-Uridinyl and Pseudouridinylradical for the

Study of Oxidative Damage in RNA

by

Raziya Shaik

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Chemistry

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The University of Toledo
December 2013
Reactive oxygen species (ROS), which are generated from various sources, are very deleterious in nature. ROS damage biological components like DNA, RNA, proteins and lipids through liberation of free radicals. A lot of research has focused on DNA damage through oxidation. Studies on RNA oxidation have, however, received much less interest until the past decade. RNA oxidation was identified to play a significant role in the etiology of neurological diseases such as Alzheimer’s and Parkinson’s.

The goal of this project is to develop the tools required for understanding the mechanisms involved in oxidative damage to RNA at a molecular level. An approach was taken which involved the generation of site specific radicals on nucleosides. Two nucleosides, uridine and pseudouridine, which are specifically found in RNA, were considered for this work. This study focuses on the generation of C5'-nucleosidylradicals from photolabile radical precursors and understanding the mechanisms of formation of radical derived products. For this purpose, syntheses were designed for the preparation of both C5'-pivaloyl substituted uridine and pseudouridine. C5'-pivaloyluridine was successfully synthesized.
and utilized for the generation of the C5'-uridinylradical through Norrish type I photocleavage. Photolyses were carried out under anaerobic and aerobic conditions in the presence and absence of hydrogen atom donors. Product formation was analyzed by HPLC and mass spectrometry. The formation of the reduction product was observed in the presence of a hydrogen atom donor. Along with the reduction product, base elimination was observed under all photolysis conditions. The synthesis of the C5'-pseudouridinylradical precursor is in progress.
I dedicate this dissertation to my parents
Acknowledgements

I express my sincere thanks to my advisor and mentor Dr. Amanda C. Bryant-Friedrich, who allowed me to enjoy working in her research group and her valuable scientific and personal experience. Her continued guidance helped me to grow as a very good chemist.

I would like to thank all the members of my commission of my final exam Dr. Tim Mueser, Dr. Viranga Tillekeratne, Dr. Dragon Isailovic for their support and guidance throughout my research.

I want to thank all my lab members Dr. Suaad Abdallah, Dr. Nicholas Jay Amato, Dr. Ting Wang Dr. Buthina Abdallah, Dr. Fernand Mel Bedi and Paul Oshule for sharing their knowledge and helping me to grow as a better person. Special thanks to Matthew Ellis and Matthew Starr for working with me on the synthesis of the C5'-Uridinyl radical precursor. Thanks to Sierra Arndt for working with me in the initial stages of the C5'-Pseudouridinyl radical precursor synthesis.

I am greatly thankful to my parents for their belief in me, and always supporting me in every stage of my life. Without their support I will never furnish myself in research. I also wanted to thank my dear friend Prasad Gobburi for all his encouragement and support during my PhD program.
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>Carbon-13 nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>dA</td>
<td>Deoxyadenosine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIAD</td>
<td>Diisopropyl azadicarboxylate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
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<tr>
<td>L-selectride</td>
<td>Lithium tri-sec-butylborohydride</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
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</tr>
<tr>
<td>NADPH</td>
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<td>$p$-Toluenesulfonic acid</td>
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<td>TBAF</td>
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<td>Trifluoroacetic acid</td>
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xxiii
Chapter 1

Introduction

1.1. Sources of Reactive Oxygen Species (ROS)

ROS are metabolites of molecular oxygen containing unpaired electrons. Reactive oxygen species include hydroxyl radicals (OH’), superoxide anions (O₂’), superoxide radicals (O₂⁻) and hydrogen peroxide (HOOH). They are produced from endogenous and exogenous sources. Higher levels of ROS are a natural phenomenon during ageing and have severe damaging effects. Cells are constantly exposed to ROS on a daily basis. These cells fight most of the negative effects of ROS, however, when defense systems are weakened, we
face the consequences of these reactive metabolites. Figure 1-1 shows some common endogenous and exogenous sources of ROS.

**Exogenous Sources**

![Figure 1-2: Damage products obtained from different wavelengths of radiation observed in DNA. “Kielbassa,C., Wavelength dependence of oxidative DNA damage induced by UV and visible light, Carcinogenesis, 1997, 18, 4, 811, by permission of Oxford University Press”](image)

Exogenous sources of ROS include various environmental toxins, such as, chemotherapeutic agents, smoking, ultraviolet and other types of ionizing radiation. UV radiation comes from sunlight, and has been determined to be a genotoxic agent. It causes permanent damage to biomolecules like DNA and RNA by creating various damage products. The extent and type of damage that occurs depends upon the wavelength of radiation (Figure 1-2).
Damage can occur either by direct or by indirect mechanisms. In the direct effect of ionizing radiation, energy is directly absorbed by the nucleobases to produce adducts like cyclobutane dimers. UVB radiation produces this type of damage (Figure 1-3).\textsuperscript{2,3} In the indirect mechanism, other cellular components of the body absorb radiation, attacking the nucleic acids, proteins and other cellular components. In addition to UV radiation, other sources, for example smoking and ionizing radiation severely damage cells and ultimately lead to cell death. Furthermore, chemotherapeutic agents damage cancer cells by increasing the production of ROS which weakens the body’s defense systems thereby damaging healthy cells.

**Endogenous Sources**

The human body constantly produces reactive oxygen species and other free radicals from enzymatic and non-enzymatic metabolic processes (Figure 1-1). Studies report that the majority of intracellular ROS production is derived from the mitochondria.\textsuperscript{4} Free radicals such as superoxide radicals are produced during oxidative phosphorylation in the mitochondria. Additionally, inflammatory cytokines, which are released during inflammation, are responsible for producing ROS by activating various stimuli.

![Figure 1-3: Photoproducts observed in RNA](image-url)
For example, increased production of ROS is observed in asthma which is a chronic inflammatory disease. Leucocytes generated in response to inflammation, release ROS, mainly superoxide radicals which, are converted to $\text{H}_2\text{O}_2$ by superoxide dismutase. Hydroxyl radicals are then generated from $\text{H}_2\text{O}_2$ by the Fenton reaction (Figure 1-4). Another endogenous source is peroxisomes, which are subcellular components mainly involved in carrying out oxidative processes. In addition to the above mentioned ways, there are many other cellular processes that generate ROS and other free radicals. Some ROS, such as hydroxyl radicals and superoxide anions, are extremely unstable and have a tendency to attack cellular components such as DNA, RNA, proteins and lipids. In this process more free radicals are generated which can challenge the integrity of the cell. However, cells have developed several defense mechanisms to counteract some of the damage caused by ROS. The impact of ROS production largely depends on the balance between ROS levels and the ability of the antioxidant defense mechanisms to modulate their reactivity. Enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase convert reactive free radical species into neutral species (Figure 1-4). For

Figure 1-4: Endogenous ROS production and antioxidant mechanisms of the cell\(^5\)
example, in Figure 1-4, superoxide radicals undergo one electron reduction to form H$_2$O$_2$ by SOD. Catalase breakdowns H$_2$O$_2$ to H$_2$O and O$_2$. These defense systems are however not completely efficient. Hence, there is always oxidative damage occurring to biomolecules. Increased generation of ROS causes oxidative stress which plays a major role in the pathology of several diseases including cancer and neurological disorders. Therefore, it is imperative to understand the mechanisms for the formation of radical derived products in biological systems.

1.2. Interaction of ROS with RNA

![Diagram of 8-oxoguanosine]

**Figure 1-5:** RNA oxidation product identified in *in vivo* studies

Similar to DNA, reactive oxygen species can cause oxidative damage to RNA. RNA oxidation plays a major role in the development of neurodegenerative diseases such as Alzheimer’s (AD) and Parkinson’s (PD).$^{7,8}$ Recently RNA oxidation gained a great deal of interest due to its demonstrated impact on the pathogenesis of neurological diseases.$^9$ Sometime ago, RNA oxidation was considered to be unimportant, because of the high turnover rate of RNA and its shorter life span. However, studies indicate that RNA is more susceptible to oxidation than DNA, because RNA is largely single stranded and is not protected by hydrogen bonding$^{10}$ which is a primary contributor to the stability of DNA
towards oxidation. Studies also indicate that the modified RNA nucleoside, 8-oxoguanosine (Figure 1-5), can be utilized as a biomarker for RNA oxidation. Quantification of 8-oxoguanosine in healthy human urine samples by HPLC-MS/MS shows the vulnerability of RNA to oxidative damage.\textsuperscript{11,12} As RNA plays a major role in protein synthesis, any damage to RNA directly impacts protein synthesis. A study by Shan’s research group showed that oxidized mRNA produces defective proteins leading to loss of normal protein levels.\textsuperscript{7,13} Based on Shan’s and Nunomura’s research on RNA oxidation, it can be hypothesized that RNA oxidation poses a challenge to the fidelity of protein synthesis. There is growing evidence that RNA oxidation is a major contributor to the development of neurological disorders like AD, to support this hypothesis studies by several research groups emphasize the importance of understanding RNA oxidation to find better treatment options for diseases like AD.

<table>
<thead>
<tr>
<th>Why is RNA oxidized?</th>
<th>How is RNA oxidized?</th>
<th>What happens after RNA oxidation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single stranded</td>
<td>Increased levels of ROS</td>
<td>Translation errors</td>
</tr>
<tr>
<td>Abundant in cells</td>
<td>Decreased antioxidant activity</td>
<td>Defective proteins</td>
</tr>
<tr>
<td>Distribution</td>
<td>Neurotoxins</td>
<td>Cell death</td>
</tr>
</tbody>
</table>

Table 1.1: RNA oxidation and consequences\textsuperscript{8}

1.3. RNA oxidation and disease relevance

As mentioned above, oxidative damage to RNA is an important factor when considering the pathology of AD and Parkinson’s disease. Reactive oxygen species in AD are generated in the neuronal cellular bodies and have very slow diffusion rates, and are therefore localized in neurons where they are responsible for oxidative stress. Studies show that in
AD patients, cytoplasmic RNA undergoes oxidation at a higher level than mitochondrial DNA.\textsuperscript{10} In Nunomura’s work the formation of 8-OHG and 8-OHdG was identified by immunostaining in brain tissue samples from AD patients. They observed higher immunoreaction in the cytoplasm where RNA is mainly concentrated. These data were compared to the oxidation of RNA in the brain tissues of non-demented patients of different ages. The observed pattern for immunoreaction in non-demented patients was similar to AD patients but with reduced intensity. Therefore, it is proposed that cytoplasmic RNA undergoes higher rates of oxidation in AD patients than DNA. Shan’s group later identified the oxidized RNA species observed in brain samples of AD. Their study showed significant amounts of poly (A)$^+$ mRNA oxidation in AD. Abnormal protein processing was observed in cells in which oxidized RNA was formed. Therefore the study concluded that RNA oxidation is a major contributor to AD.\textsuperscript{13}
In the continuation of this work, Shan’s group quantified the extent of mRNA oxidation by isolating the oxidized mRNA species from nonoxidized mRNA by immunoprecipitation using 8-OHG antibodies (Figure 1-7). The abundance of oxidation was compared with age matched normal individuals. About 30-70% of the mRNA was found to be oxidized in brain samples of AD patients. This is considered to be high when compared to normal individuals.⁷

![Figure 1-7: (A): Southern blot analysis of oxidized (O) and nonoxidized mRNA (N) mRNA in the brain samples of AD and normal individuals. (B): Densitometric analysis of southern blot results showed the 52.3 ± 6.23% oxidized mRNA, while only 1.78 ± 0.58% mRNA oxidized in normal individuals. “Reprinted from Shan, X., Quantification of Oxidized RNAs in Alzheimer’s Disease, Neurobiology of Aging, 2006, 27, 657-662, Copyright (2013), with permission from Elsevier”⁷](image)
Quantification of oxidized nucleosides using high performance liquid chromatography-mass spectrometry has been largely responsible for the recent advancement of our understanding of the extent of oxidation in nucleic acids. These analyses were performed on human urine samples. This technique was found to be very useful, because urine samples can be injected into reverse phase HPLC without pretreatment which eliminates tedious sample preparation. Analysis of urinary samples by this method was further developed by Andreoli’s group to improve the separation and identification of oxidized products.\textsuperscript{12} Quantification of oxidized nucleosides by HPLC-ESI showed about 23% ribonucleoside oxidation, while only 13% deoxyribonucleoside oxidation was observed.\textsuperscript{11} These studies signify the extent of RNA oxidation compared to DNA.

In addition to AD, mRNA oxidation was also observed in other disease conditions such as atherosclerosis. As in AD, the oxidation of cytoplasmic RNA has been identified as a major contributor for disease development.\textsuperscript{14} The role of RNA oxidation in several diseases can be contributed to its role in the formation of damaged or modified proteins.

\textbf{1.4. Studies on RNA oxidation}

Studies show that the production of ROS in the body has many detrimental effects. Therefore, it is important to understand damage caused by the generation of ROS by studying the formation of radical derived products. Many research groups including ours focus on understanding mechanisms related to oxidative damage to DNA. From the above studies, it is known that RNA equally undergoes oxidative damage. Therefore our group is also interested in studying the damage caused by the generation
of radicals in RNA. We are mainly interested in understanding the damage that occurs to the DNA/RNA through the generation of sugar radicals. Because strand breaks in DNA/RNA are a consequence of the generation of sugar radicals, it is essential to understand their mechanisms of degradation. Most studies involving the generation of sugar radicals, utilize the site specific generation of radicals at a desired position. This can be achieved by the synthesis of suitable radical precursors that can specifically deliver the radical of interest. Radical precursors are very beneficial to guide our understanding of the products derived from a radical at a particular position on the nucleoside, either base or sugar. Furthermore, by this method most of the undesirable products coming from other irrelevant pathways can be eliminated. This makes the identification of unknown products very straightforward.

In the current study, our research focuses on understanding the fate and mechanism of formation of the products obtained from the generation of the C5'-uridinyl radical and pseudouridinyl radical. A few studies have been reported on RNA oxidation through independent generation of radicals at specific positions on the nucleoside. The most recent work was reported by Greenberg involving the generation and fate of 5, 6-dihydrouridin-6-yl radical\textsuperscript{15,16} (Scheme 1-1). This study involves site specific generation of the 5,6-dihydro-uridin-6-yl radical through Norrish type I photocleavage of the radical precursor. The C6-radical then undergoes intramolecular hydrogen abstraction from the C2'-position of the adjacent nucleotide leading to strand scission. An important observation should be made here. In the case of DNA, intramolecular hydrogen abstraction via the analogous radical occurs at the C1'-hydrogen atom,
however, in RNA the C2'-H bond dissociation energy is reduced due to the presence of the C2'-OH group. This signifies that the reactivity of RNA is different from that of DNA. Work from Giese’s group focused on the study of the differences in reactivity of the C4' radicals in RNA and DNA (Scheme 1-2). Their study indicated that under aerobic conditions, the product composition from the C4'-radical in RNA is strongly influenced by the presence of the C2'-OH group. They showed that the rate of strand cleavage in RNA is three times slower than in DNA.\textsuperscript{17}

**Scheme 1-1:** Generation and fate of the 5,6-dihydouridin-6-yl
This study further emphasized that the reactivity of the ribose moiety is different from deoxyribose due to the C2'-OH group. These studies also indicate the need for more studies in RNA to get more mechanistic understanding of the ribose sugar radicals in this context. Though a lot of studies focus on the oxidation of DNA, both at the sugar and the nucleobase, the fate of C5'-radicals in deoxyribose sugars has not been widely explored. Generation of radicals at all other positions on the sugar lead to nucleobase elimination. Most of the research on the C5'-radical in nucleosides through site specific generation comes from Chatgilialoglu’s group.

Scheme 1-2: Fate of C4'-ribose radicals\textsuperscript{17}
Their studies showed the formation of cyclized products through radical generation at C5' in purine containing nucleosides (Scheme 1-3). In the case of adenosine, under anaerobic conditions, the main product observed was C5', 8-cyclo-2'-deoxyadenosine (18a and 19a). The analogous cyclized product (18b and 19b) was also observed with 2'-deoxyguanosine through independent generation of the C5'-radical. Under aerobic conditions, the predominant product observed was the C5'-nucleoside aldehyde (22). Other studies on C5' oxidation were carried out by treating oligonucleotides with antibiotic chemotherapeutic agents such as neocarzinostatin, bleomycin and calicheamicin. So far, only one study from Chatgilialoglu’s lab utilized the independent generation of the C5'-radical through the synthesis of a radical precursor.
These studies are in the preliminary stages with no further progress being reported. Therefore, it is essential to put more effort into understanding the nature of the C5' radical and its contribution to total oxidative damage to RNA. For these studies uridine is the chosen nucleoside. The reason for this is pretty straightforward, uridine is the only nucleoside that is found in RNA and not in DNA. Hence studies utilizing this nucleoside would be more relevant for understanding damage to RNA. Therefore, the goals of this work involve the synthesis of suitable radical precursors for the generation of the C5'-uridinylradical and determination of radical degradation products by chromatography and spectroscopic methods in addition to probing the mechanisms involved in the formation of the degradation products derived from this reactive intermediate.

Scheme 1-4: Outline for the generation and fate of the C5'-uridinylradical

RNA undergoes post transcriptional modifications that lead to the formation of nearly 100 different nucleoside modifications.\textsuperscript{23,24}
These modified nucleosides are found in all types of RNA from animal, plant and bacterial origin. Among these modified nucleosides, pseudouridine ($\Psi$) is the most frequently occurring post-transcriptional modification found in humans. It is considered the fifth nucleoside due to its ubiquitous nature. Pseudouridine is a C-glycoside isomer of uridine that distinguishes itself from uridine by the presence of an additional hydrogen bond donor (Figure 1-8). This additional feature plays an important role in increasing RNA stacking in single stranded and duplex regions and favoring the C3'-endo conformation of the ribose moiety. The orientation of the nucleobase is fixed in an anti conformation, which increases the rigidity of the molecule. Consequently, other nucleosides in the sequence orient themselves accordingly which improves the stability of the RNA structure. The presence of a water bridge causes stabilization by replacing the C5-H.....O$_W$ interaction in uridine by a stronger N1-H.....O$_W$ in pseudouridine through pseudouridylation (Figure 1-9). The lowered flexibility of the pseudouridine moiety renders the diphosphate backbone

**Figure 1-8**: Structural differences between uridine and pseudouridine
in its vicinity very rigid. This alternatively improves the cooperative base stacking of neighboring nucleosides, which contribute to the overall stability of the RNA structure.

![Diagram of pseudouridine nucleotide with a water molecule.](image)

**Figure 1-9**: Hydrogen bonding of pseudouridine nucleotide with a water molecule. “Reprinted from Charette, M., *Pseudouridine in RNA: What, where, how and why*, IUBMB Life, 2000, 49, 341-351, with permission from John Wiley and Sons”.

The biosynthesis of pseudouridine is carried out by a class of enzymes known as pseudouridine synthases through the isomerization of uridine to pseudouridine in oligonucleotides. Though the mechanism of this isomerization is not completely understood, it was proposed that a glycosyl bond must be cleaved, followed by the rotation of the uracil base and formation of a C-C bond while the substrate is enzyme bound. Santi’s group proposed a mechanism involving a Michael adduct formed between the enzyme and the substrate (*Scheme 1-5*). However, this is not a completely proven mechanism.
1.5. Pseudouridine and disease relevance

Studies show that urinary levels of pseudouridine are high in AD patients. From the above discussion, it is known that RNA oxidation plays a major role in the development of AD. Because pseudouridine is a modified nucleoside in RNA, it can be assumed that there is a connection between oxidative stress and higher urinary levels of pseudouridine. Therefore, understanding the behavior of pseudouridine by subjecting it to oxidative conditions might provide some key information about the role of this nucleoside in RNA related processes. It is also possible that under oxidative conditions,
the biosynthesis of pseudouridine is higher, and therefore urinary levels in AD patients are related to this fact. It may be possible that under oxidative stress, uridine is isomerized to pseudouridine. If this is the case then pseudouridine would be more stable than uridine under these conditions. Studies show that pseudouridine plays a regulatory role in maintaining the structure of RNA. If we consider these studies, it can be presumed that isomerization of uridine to pseudouridine may be a defense mechanism to protect the cell from oxidative stress. To investigate these possibilities, pseudouridine will also be subjected to oxidative damage. Therefore, the second part of this study involves the synthesis of a C5'-pseudouridinylradical precursor and studying the fate of the photochemically generated radical (Scheme 1-6). Comparing the behavior of the uridine and pseudouridine C5'-ribosyl radicals under the same conditions will provide invaluable information to address some of the above mentioned predictions.

**Scheme 1-6:** Outline for the generation and fate of the C5'-pseudouridinylradical
Chapter 2

Experimental Methods

All organic reactions were carried out under inert standard laboratory conditions under an argon atmosphere with magnetic stirring using oven-dried glassware unless otherwise indicated. Where water and oxygen exclusion were necessary, air and moisture-sensitive liquids were transferred via syringe through rubber septa.

2.1. Materials

All chemicals and reagents were commercially available, and were of the highest grade and used without further purification unless otherwise stated. Deuterated solvents for NMR were purchased from Cambridge Isotope Laboratories. All other solvents were purchased from Acros Organics and Fisher Scientific. For chromatographic separation, HPLC grade solvents were purchased from PHARMACO-AAPER. Anhydrous THF was dried over activated alumina. Uridine and pyridinium p-toluenesulfonate were purchased from Acros Organics. Potassium cyanide, t-butyldimethylsilyl cyanide and t-BuLi (1.7 M in pentanes) were purchased from Sigma-Aldrich. 18-Crown-6 was also purchased from Avacado Research Chemicals. 3-Hydroxypicolinic acid and ammonium citrate dibasic were purchased from Fluka. Deionized water was purified with a PURELAB® Ultra Water
Purification Systems. All reagents for oligonucleotide synthesis were purchased from Glen Research.

2.2. Analytical Methods

All synthesized products were characterized by NMR and MS spectrometry.

$^1$H NMR

$^1$H NMR spectra were acquired on an Inova-600 or Avance-600 NMR Spectrometer in CDCl$_3$ or CD$_3$OD. The $^1$H NMR data are presented as follows: Chemical shift, integration, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet) and coupling constants (in Hertz). The protons for the carbon centers of the furanose ring and the nucleobase of uridine and pseudouridine are designated as shown in the figure below.

![Figure 2-1: The proton assignments of the sugar and the nucleobase moieties in uridine and pseudouridine](image)

$^{13}$C NMR

$^{13}$C NMR spectra were recorded on an Inova-600 and Avance-600 Nuclear Magnetic Resonance spectrometer in CDCl$_3$, CD$_3$OD or (CD$_3$)$_2$SO. The spectra are $^1$H- broadband-decoupled.
Mass spectrometry

Mass spectrometric analyses of synthesized products were performed using ESI and MALDI-TOF MS.

ESI-MS

Low resolution mass spectra were obtained on an Esquire-electrospray ionization mass spectrometer (Bruker Daltonics, Bermen, Germany) operated in positive ion mode. Samples were prepared by dissolving analyte in methanol or another suitable solvent.

MALDI-TOF MS

MALDI-TOF measurements were conducted using a MALDI-TOF UltrafleXtreme (Bruker Daltonics, Billerica, MA) mass spectrometer operating in positive-ion, reflectron mode. External calibration was used for the mass range of 0 to 600 m/z. For small molecule analysis dihydroxybenzoic acid (DHB) was used as matrix. The matrix was prepared by dissolving DHB (~2 mg/ 50 µL) in 70 % of A (0.1% TFA in H$_2$O) and 30 % B (CH$_3$CN). One µl of this matrix solution was spotted on a MALDI plate followed by 1µl of sample. The sample was prepared by dissolving the analyte in water or 1:1 v/v CH$_3$CN: H$_2$O. MALDI signals were observed corresponding to [M+H$^+$] ions of the measured analyte. A method utilizing 38% laser and 200 shots /scan was applied as standard settings for analysis.

High Resolution Mass Spectrometry
High resolution mass spectrometry (HRMS) was performed on a mass spectrometer located in the Mass Spectrometry and Proteomics Facility at The Ohio State University, Columbus, Ohio.

### 2.3. Chromatographic Methods

#### Thin layer chromatography

Analytical TLC was carried out on Silicycle® silica gel 60 F254 aluminum backed plates. Product spots were visualized by UV light at 254 nm and/or staining the TLC plate with anisaldehyde dip.

#### Flash Chromatography

Purification of organic compounds was performed on a Biotage SP4 automated chromatography system with an in-line variable wavelength detector. Biotage SNAP cartridge silica gel pre-packed columns (KP-Sil) were used for purification. Nucleosides were detected at 254 nm and monitored at 260 nm.

#### High Performance Liquid Chromatography

HPLC was performed on a Dionex Ultimate 3000 HPLC system equipped with a variable wavelength detector. Reversed-phase HPLC was used for the analysis of photolysates. The solvent system contained, solvent A: 50 mM TEAA buffer, pH = 7.0 or solvent D: water, solvent B: acetonitrile.

### 2.4. Equipment and Devices
Solvent purification system- *Innovative Technology* PS-MD-2 Pure Solvent System

Rotary evaporator- *Heidolph Collegiate Brinkmann* rotovap

High vacuum pump- *Edwards RV3*

Speed vacuum - *Thermoelectron Savant* DNA120

Micro centrifuge – *Thermo Sorvall Legend* Micro 21

Vortex mixer- *Fisher Scientific*

Thermal mixer- *Eppendorf* Thermomixer

pH meter- *Fisher Accumet Basic AB15*

Pipettes- *Eppendorf* Series 2100
2.5. Synthesis of C5-Pivaloyl uridine

2.5.1. 2-tert-butyldimethylsilyloxy-2-((3aS,4R,6R,6aS)-tetrahydro-4-(3,4-dihydro-2,4-dioxopyrimidin-1(2H)-yl)-2,2-dimethylfuro[3,4-d][1,3]dioxol-6yl)acetonitrile (34)

Uridine-5'-aldehyde (1.73 g, 6.13 mmol), 18-crown-6 (5.05 mg, 0.02 mmol) and potassium cyanide (6.5 mg, 0.10 mmol) were coevaporated with anhydrous dichloromethane (3 x 20 ml). To this, anhydrous THF (40 mL) was added followed by the addition of tert-butyldimethylsilyl cyanide (2.16 g, 15.3 mmol) as a solution in anhydrous THF (10 mL). The resulting solution was left to stir at rt for 16 h. The reaction was quenched by the addition of water (50 mL) and the product extracted with ethyl acetate (3 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and the solvent removed to deliver the crude product. Purification by flash chromatography with 3:97 methanol: dichloromethane, gave a mixture of diastereomers in 1.25:1 ratio (as determined by NMR) as a colorless oil (70% yield). $^1$H NMR (CDCl$_3$): $\delta$ 10.23 (1H, s, NH), 10.16 (1H, s, NH), 7.44 (1H, d, $J = 8.1$), 7.28 (1H, d, $J = 7.8$), 5.82 (1H, d, $J = 2.4$), 5.76 (2H, t, $J = 8.4$), 5.60 (1H, m), 5.07 (1H, dd, $J = 6.5$ Hz, $J = 1.6$ Hz), 4.92 (3H, dd, $J = 6.5$ Hz, $J = 2.6$ Hz), 4.90 (1H, dd, $J = 6.6$ Hz, $J = 3.2$ Hz), 4.87 (1H, dd, $J = 6.3$ Hz, $J = 3.2$ Hz), 4.75 (1H, d, $J = 6.1$ Hz), 4.72 (1H, d, $J = 8.1$ Hz), 4.32 (1H, dd, $J = 5.9$ Hz, $J = 3.2$ Hz), 4.25 (1H, dd, $J = 7.9$ Hz, $J = 3.3$ Hz), 1.57 (3H, s), 1.54 (3H, s), 1.35
(3H, s), 1.33 (3H, s), 0.92 (9H, s), 0.88 (9H, s), 0.23 (3H, s), 0.19 (3H, s), 0.15 (3H, s), 0.11 (3H, s).

$^{13}$C NMR (CDCl$_3$): δ 163.9, 163.7, 150.5, 150.3, 143.6, 141.8, 118.6, 117.8, 115.0, 114.6, 103.1, 102.9, 96.8, 94.2, 88.5, 87.7, 84.29, 84.24, 82.1, 80.7, 63.4, 62.8, 27.1, 27.0, 25.6, 25.5, 25.3, 25.2, 18.2, 18.1, -4.8, -5.0, -5.1, -5.2

HRMS [M +Na]$^+$: calc. for C$_{19}$H$_{29}$O$_6$N$_3$Si 446.1723, found 446.1725.

2.5.2. 1-((3aS,4R,6R,6aS)-4-(1-tert-butyldimethylsilyloxy-2-imino-3,3-dimethylbutyl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxol-6-yl)pyrimidine-2,4(1H,3H)-dione (35)

![Chemical Structure](image)

To a solution of 34 (0.20 gm, 0.05 mmol) in anhydrous THF at – 78 °C (8 mL), was added $t$-BuLi (1.1 mL, 1.9 mmol, 1.7 M in pentane). The resulting solution was stirred for 15 min and quenched by the addition of water. The mixture was then allowed to warm to room temperature and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were washed with water until the aqueous layer became neutral and finally washed with saturated sodium chloride. The ethyl acetate was removed in vacuo to deliver crude imine 35.

2.5.3. 1-((3aS,4S,6R,6aS)-4-(1-tert-butoxy-3,3-dimethyl-2-oxobutyl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxol-6-yl)pyrimidine-2,4(1H,3H)-dione (36)
Compound 35 was subjected to hydrolysis by the addition of 10 ml of THF/ H₂O/ 2N HCl (40: 20: 1). The solution was allowed to stir at rt for 5 hours followed by extraction with ethyl acetate (3 × ~20 ml). The combined organic layers were washed with saturated sodium chloride and dried over anhydrous sodium sulfate. Finally, the solvent was removed in vacuo to give a crude brownish oil. Purification by flash chromatography with ethyl acetate: hexanes (1:2) gave 36 as a mixture of isomers in a 1.5:1 ratio as determined by NMR. (yield 69%). ¹H NMR (CDCl₃): δ 9.70 (1H, s, NH), 9.36 (1H, s, NH), 8.06 (1H, d, J = 8.3 Hz), 7.30 (1H, d, J = 8.1 Hz), 6.00 (1H, d, J = 3.4 Hz), 5.88 (1H, d, J = 2.9 Hz), 5.76 (1H, dd, J = 8.2 Hz, J = 1.8 Hz), 5.72 (1H, d, J = 8.1 Hz), 4.97 (1H, dd, J = 6.2 Hz, J = 4.2 Hz), 4.93 (1H, d, J = 4.4 Hz), 4.92 (1H, d, J = 2.2 Hz), 4.85 (1H, m), 4.80 (1H, s), 4.74 (1H, dd, J = 6.8 Hz, J = 2.9 Hz), 4.63 (1H, dd, J = 5.9 Hz, J = 3.7 Hz), 4.28 (1H, t, J = 4.2), 1.60 (3H, s), 1.51 (3H, s), 1.34 (3H, s), 1.30 (3H, s), 1.22 (9H, s), 1.16 (9H, s), 0.90 (9H, s), 0.88 (9H, s), 0.10 (3H, s), 0.06 (3H, s), 0.01 (3H, s), 0.01 (3H, s)

¹³C NMR: δ 213.2, 211.2, 163.6, 163.3, 150.5, 150.3, 141.3, 141.1, 115.0, 114.2, 103.1, 102.2, 92.7, 91.4, 86.0, 85.9, 85.5, 83.9, 81.7, 79.2, 75.3, 72.9, 43.9, 43.4, 27.5, 27.39, 27.35, 26.9, 26.0, 25.9, 25.5, 25.3, 18.5, 18.4, -3.9, -4.1, -4.3, -5.2

HRMS [M + Na]⁺: calc. for C₂₃H₂₈O₇N₂Si 505.2346, found 505.2356.
2.5.4. 1-((3aS,4R,6R,6aS)-tetrahydro-4-(1-hydroxy-3,3-dimethyl-2-oxobutyl)-2,2-
dimethylfuro[3,4-d][1,3]dioxol-6-yl)pyrimidine-2,4(1H,3H)-dione (121)

To 36 (0.56 g, 1.16 mmol) in anhydrous THF (10 ml) at 0 °C tetra-n-butylammonium
fluoride (1.75 ml, 1.75 mmol, 1 M in THF ) was added. The ice bath was removed after 15
min and the reaction mixture was stirred at rt for 1 hour. The solvent was removed to give
crude 121. This mixture was purified by flash column chromatography with 1:1 ethyl
acetate: hexanes to give a mixture of two diastereomers in 1.5: 1 ratio (yield 84 %).

$^1$H NMR (CDCl$_3$): δ 7.67 (1H, d, $J = 8.1$ Hz), 7.43 (1H, d, $J = 8.1$ Hz), 5.94 (1H, d, $J = 3.7$
Hz), 5.90 (1H, d, $J = 2.9$ Hz), 5.77 (1H, d, $J = 8.3$ Hz), 5.75 (1H, d, $J = 8.1$ Hz), 5.03 (1H,
dd, $J = 6.2$ Hz, $J = 2.8$ Hz), 4.83 (2H, m), 4.75 (2H, m), 4.67 (1H, m), 4.55 (1H, m), 1.60
(3H, s), 1.55 (3H, s), 1.36 (3H, s), 1.31 (3H, s), 1.24 (9H, s), 1.22 (9H, s).

$^{13}$C NMR (CDCl$_3$): δ 214.91, 213.52, 163.63, 163.45, 150.66, 150.48, 140.62, 140.48,
114.84, 114.60, 103.21, 103.13, 91.86, 91.75, 85.00, 84.92, 83.97, 83.86, 81.01, 80.04,
73.66, 73.55, 43.58, 43.17, 27.48, 27.42, 27.36, 27.30, 27.13, 27.07, 26.57, 26.50, 25.51,
25.44, 25.43 and 25.36.

HRMS [M + Na]$^+$: calc. for C$_{17}$H$_{24}$O$_7$N$_2$ 391.1481, found 391.1490.
2.5.5. 1-((2R,3S,4R,5R)-tetrahydro-3,4-dihydroxy-5-(hydroxyl-5-tert-butylcarbonyl-methyl)furan-2-yl)pyrimidine-2,4(1H,3H)-dione (27)

To 121 (0.24 g, 0.65 mmol), 20 mL of 25% acetic acid were added and the resulting suspension was refluxed for 1 hour. After the reaction cooled to rt, the solvent was removed under vacuum to give pure 27 as two isomers in a 2:1 ratio (as determined by HPLC) (yield 75%).

$^1$H NMR (DMSO-d$_6$): δ 11.32 (1H, s), 7.97 (1H, d, $J = 7.8$), 7.81 (1H, d, $J = 7.8$), 5.63-5.81 (4H, m), 5.48 (1H, s), 5.38 (1H, s), 5.29 (1H, s), 5.18 (1H, s), 4.63 (1H, dd, $J = 3.4$), 4.58 (1H, dd, $J = 12.0$), 4.16-4.27 (2H, m), 4.00-4.05 (3H, m), 1.13 (9H, s), 1.09 (9H, s).

$^{13}$C NMR: δ 213.8, 213.6, 163.6, 162.9, 151.6, 151.2, 141.4, 141.2, 102.4, 102.0, 88.2, 87.9, 85.2, 85.0, 73.8, 73.3, 70.8, 69.8, 67.4, 62.4, 43.7, 43.4, 26.7, 26.2.

HRMS [M +Na]$^+$: calc. for C$_{14}$H$_{20}$O$_7$N$_2$ 351.1168, found 351.1151.

2.5.6. 1-((3aS,4R,6R,6aS)-4-(tert-butyldimethylsilyloxy)methyl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxol-6-yl)pyrimidine-2,4 (1H,3H)-dione (37)
2,6-Lutidine (0.1 ml, 0.87 mmol) was added to a solution of 2',3'-isopropylideneuridine (0.10 g, 0.35 mmol) in dry THF (3.0 ml) at 0 ºC. After 15 min, TBSOTf (0.18 ml, 0.78 mmol) was added and the solution brought to room temperature and stirred for 2 hours. The product was extracted with ethyl acetate. The solvent was removed under vacuum and the compound was purified using silica gel column chromatography with 50% ethyl acetate in hexanes (yield 72%).

$^1$H NMR (CDCl$_3$): 9.13 (NH, s), 7.71 (1H, d, $J = 8.1$ Hz), 5.99 (1H, d, $J = 2.7$ Hz), 5.69 (1H, dd, $J = 8.1$ Hz, $J = 2.0$ Hz), 4.77 (1H, dd, $J = 6.1$ Hz, $J = 2.9$ Hz), 4.69 (1H, dd, $J = 6.1$ Hz, $J = 2.7$ Hz), 4.33 (1H, m), 3.93 (1H, dd, $J = 11.6$ Hz, $J = 2.3$ Hz), 3.80 (1H, dd, $J = 11.5$ Hz, $J = 2.9$ Hz), 1.59 (3H, s), 1.36 (3H, s), 0.90 (9H, s), 0.10 (3H, s), 0.09(3H, s).

$^{13}$C NMR (CDCl$_3$): 163.48, 150.32, 140.77, 114.30, 120.39, 92.09, 86.86, 85.58, 80.45, 63.55, 27.47, 26.04, 25.55, 18.53, -5.24 and -5.35.

HRMS [M +Na]$^+$: calc. for C$_{18}$H$_{30}$O$_6$N$_2$Si 421.1771, found 421.1776.

2.6. Photolysis of C5'-pivaloyluridine

Photolysis experiments were carried out in 4.5 cm × 10 mm quartz cuvettes (Sigma Aldrich, St. Louis, MO) using an Oriel 500 high pressure mercury arc lamp (Newport, Irvine, CA) fitted with an IR filter, focusing lens and cut off filter. All photolyses were carried out at ≥
320 nm and the temperature during photolysis was maintained between 15-25 °C using a Peltier PTP-1 single cell temperature control system. (Varian, Palo Alto, CA). Initial photolysis experiments were carried out at 15, 30, 45 and 60 min. Sixty min time intervals were used for later experiments. The photolysis mixtures obtained were either subjected to evaporation for a fixed amount of time or directly injected onto an analytical RP-HPLC column. A Thermo Hypersil-keystone BDS Hypersil C18 column (4.6 × 250 cm, 5 µm) was used for the separation of photolysis products with UV detection at 254 nm.

Gradient 1: A, 50 mM TEAA, pH = 7.0; B = Acetonitrile: 0 to 10% B in 12 min, 10 – 32% B in 25 min. Flow rate used was 1.0 mL/ min.

Gradient 2: Same as above except 50 mM TEAA was replaced with water.

Gradient 3: 25 to 50% B in 10 min, 50 to 75% B in 20 min, 75-95% B in 28 min. In all cases, the column was preconditioned for 10 min prior to sample injection.

2.6.1. Photochemical generation of the C5'-uridylnylradical in the presence of tri-n-butylnit tin hydride

2.6.1.1. Photolysis of compound 27

General procedure

A sample containing compound 27 in a suitable solvent was prepared and to this tri-n-butylnit tin hydride was added. The solution was purged using a stream of bubbling argon gas through polymer tubing (Western Analytical Products, Wildomar, CA) for 15 min. The sample was photolysed for the specified amount of time shown in Table 2.1 at ~15 °C. After photolysis, the crude photolysate was evaporated for 30 min to reduce to half volume and injected on an HPLC column. Fractions were collected, the solvent was removed and
the residue analyzed by mass spectrometry. Products were identified by comparison with standards by spiking the photolysate with a known concentration of each suspected product.

<table>
<thead>
<tr>
<th>Conc. of <strong>27</strong> (mM)</th>
<th>Conc. of TBTH (M)</th>
<th>Solvent (v/v)</th>
<th>Photolysis time</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.05</td>
<td>1:1 CH₃CN/H₂O</td>
<td>15, 30, 45 and 60 min</td>
<td>1</td>
</tr>
<tr>
<td>0.37</td>
<td>0.185</td>
<td>1:1 CH₃CN/H₂O</td>
<td>15, 30, 45 and 60 min</td>
<td>1 and 2</td>
</tr>
<tr>
<td>0.37</td>
<td>0.37</td>
<td>1:1 CH₃CN/H₂O</td>
<td>60 min</td>
<td>2</td>
</tr>
<tr>
<td>0.37</td>
<td>0.185</td>
<td>CH₃CN</td>
<td>60 min</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2.1:** Conditions used for the photolysis of **27** in the presence of tri-**n**-butyltin hydride

**2.6.1.2. Photolysis of compound 36**

A sample containing compound 36 (0.37 mM) in 2:1 v/v CH₃CN:H₂O was prepared and to this tri-**n**-butyltin hydride (0.37 M) was added. This solution was purged using a stream of bubbling argon gas through polymer tubing for 15 min. The sample was photolysed for 1 hour at ~15 °C. After photolysis the crude photolysate was injected onto a RP-HPLC column (gradient 3). Fractions were collected, the solvent removed and products analyzed by mass spectrometry. Products were compared with standards by spiking the photolysate with known concentrations of the expected products.

**2.6.2. Photochemical generation of the C5'-uridinylradical in the presence of glutathione**

**General procedure**
A sample containing compound 27 in water or 10 mM phosphate buffer (~2% CH3CN) was prepared and to this glutathione was added. This solution was purged using a stream of bubbling argon gas through polymer tubing for 15 min. The sample was photolysed for 60 min at ~15 ºC. After photolysis the crude photolysate was evaporated for 5 min and injected onto the HPLC using a suitable gradient. Fractions were collected, the solvent was evaporated and the products analyzed by mass spectrometry. Products were compared with standards by spiking the photolysate with known concentrations of the expected products.

Making solutions containing different concentrations of GSH

General procedure

Weighed amounts of GSH were dissolved in a specific volume of 10 mM phosphate buffer and the pH was adjusted to 7.0 or 3.5. This glutathione solution was added to a stock solution of 27 to make the final volume. The glutathione solution was prepared in such a way that addition of GSH solution alone made up the volume required for photolysis, hence there was no need to add water or buffer. In this way issues with change pH or GSH concentration were minimized (Table 2.2).

<table>
<thead>
<tr>
<th>Conc. of 27 (mM)</th>
<th>Conc. of GSH (mM)</th>
<th>Solvent</th>
<th>Photolysis time</th>
<th>Gradient condition</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>100</td>
<td>Water</td>
<td>60 min</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>0.37</td>
<td>2, 4, 6, 8, 16, 24 and 32</td>
<td>10 mM phosphate buffer</td>
<td>60 min</td>
<td>2</td>
<td>7.0</td>
</tr>
<tr>
<td>0.37</td>
<td>2, 4, 6, 8, 16, 24 and 32</td>
<td>10 mM phosphate buffer</td>
<td>60 min</td>
<td>2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 2.2: Conditions used for the photolysis of 27 in the presence of glutathione
2.6.3. Photochemical generation of the C5′-uridinylradical in the absence of glutathione

General procedure

The same procedure was used as in previous experiments (Table 2.3).

<table>
<thead>
<tr>
<th>Conc. of 27 (mM)</th>
<th>Conc. of GSH (mM)</th>
<th>Solvent</th>
<th>Photolysis time</th>
<th>Gradient condition</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.37</td>
<td>0</td>
<td>10 mM phosphate buffer</td>
<td>60 min</td>
<td>2</td>
<td>7.0</td>
</tr>
<tr>
<td>0.37</td>
<td>0</td>
<td>10 mM phosphate buffer</td>
<td>60 min</td>
<td>2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 2.3: Conditions used for the photolysis of 27 in the absence of glutathione

2.6.4. Photochemical generation of the C5′-uridinylradical under aerobic conditions

Making 6 mM GSH

Six mM GSH was made in water and directly added to the stock solution to make the final concentration (Table 2.4).

<table>
<thead>
<tr>
<th>Conc. Of 27 (mM)</th>
<th>Conc. of GSH (mM)</th>
<th>Solvent (v/v)</th>
<th>Photolysis time</th>
<th>Gradient condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.37</td>
<td>6</td>
<td>Water</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>0.37</td>
<td>0</td>
<td>Water</td>
<td>60</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2.4: Conditions used for the photolysis of 27 under aerobic conditions in the presence/absence of glutathione

2.7. Synthesis of a Pseudouridine-C5′-Radical Precursor

2.7.1. Synthesis of 6-(tert-butyldimethylsilyloxy methyl)-4-(2, 4-di-tert-butoxypyrimidin-5-yl)-5-tetrahydro-2, 2-dimethylfuro [3, 4-d] [1,3]-dioxo-4-ol (38)
2,4-Di-tert-butoxy-5-bromopyrimidine (3.10 g, 10.0 mmol) was dissolved in 90.0 ml of dry THF and the solution was left to stir at -78 °C for 15 min. To this solution n-BuLi (9.37 ml, 15.0 mmol, 1.6 M in hexanes) was added and stirred for 1 hr and 15 min at -78 °C. 5-O-tert-butyldiphenyl-silyl-2,3-O-isopropylidene-D-ribo-1,4-lactone (4.38 g, 10.0 mmol) was then added by canulation as a solution in 15.0 ml of dry THF and the solution was left to stir for an additional 5 hrs followed by quenching with brine. The product was extracted with ethyl acetate (3 × 150 ml) and the combined organic extracts were dried over anhydrous sodium sulfate, filtered and evaporated to give an orange oily product. The substrate was obtained as a mixture of isomers in a 1:4 ratio (by $^1$H NMR). Purification was carried out using silica gel column chromatography using ethyl acetate: hexanes (15:85) to give 38 as a pure colorless oil (yield 84.1%).

$^1$H NMR (CDCl$_3$): δ 8.54 (1H, s), 8.46 (1H, s), 7.72 (3H, m), 7.68 (3H, m), 7.64 (2H, m), 7.42 (12H, m), 4.92 (2H, m), 4.87 (1H, dd, $J = 5.7$ Hz, $J = 1.3$ Hz), 4.73 (1H, m), 4.51 (1H, m), 4.36 (2H, m), 3.91 (1H, m), 3.80 (1H, d, $J = 3.9$ Hz), 3.78 (1H, m), 1.66 (9H, s), 1.63 (3H, s), 1.62 (9H, s), 1.61 (3H, s), 1.28 (3H, s), 1.23 (3H, s), 1.11 (12H, s), 1.05 (3H, s), 1.01 (3H, s).

$^{13}$C NMR (CDCl$_3$): δ 167.09, 167.05, 164.15, 164.11, 156.86, 156.60, 135.88, 135.77, 135.74, 135.71, 135.56, 132.44, 132.41, 130.35, 130.34, 130.30, 130.16, 129.96, 129.94,
128.18, 128.16, 128.09, 128.05, 127.92, 127.90, 116.44, 115.33, 115.06, 113.25, 112.30, 105.94, 100.46, 87.61, 86.18, 84.93, 82.46, 82.43, 82.38, 82.23, 82.18, 82.14, 81.26, 80.28, 80.19, 78.59, 75.97, 65.70, 63.81, 63.86, 28.78, 28.75, 28.56, 28.54, 27.00, 26.99, 26.93, 26.90, 26.77, 26.65, 25.74, 25.68, 25.38, 19.35, 19.33 and 19.20.

HRMS [M +H]⁺: calc. for C_{36}H_{50}O_{7}N_{2}Si, 651.3466; found 651.3467

2.7.2. Synthesis of 2-tert-butyldimethylsilyloxy-1-(5-((2,4-di-tert-butoxypyrimidin-5-yl) (hydroxy)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)ethanol (39)

To 38 (0.48 g, 0.73 mmol) in dry dichloromethane (28 ml), at -78 ºC was added slowly ZnCl₂ (1 ml, 1 mmol, 1.0 M solution in Et₂O). After 30 min, L-selectride (3.4 ml, 3.4 mmol, 1.0 M solution in THF) was added portion wise over 30 min. This solution was brought to room temperature and left to stir overnight. Reaction progress was monitored by TLC. The reaction was stopped by consecutive addition of EtOH (0.50 ml), H₂O (0.10 ml), 6.0 M NaOH (0.36 ml) and 30% H₂O₂ (0.36 ml) and the solution left to stir at room temperature for 30 min. The product was extracted with dichloromethane (3 × 50 ml), the combined organic extracts were washed with saturated NaHCO₃ (100 ml) and saturated NaCl (100 ml) and dried over Na₂SO₄. The solvent was removed to give an oily crude product. It was purified by column chromatography using ethyl acetate: hexanes (20:80) in 66.6% yield.
$^1$H NMR (CDCl$_3$): δ 8.30 (1H, s), 7.72 - 7.69 (4H, m), 7.45 - 7.38 (6H, m), 5.26 (1H, d, $J$ = 5.5 Hz), 4.35 (1H, dd, $J$ = 6.2 Hz, $J$ = 1.5 Hz), 4.23 - 4.30 (2H, m), 3.93 (1H, dd, $J$ = 10.5 Hz, $J$ = 3.1 Hz), 3.87 (1H, dd, $J$ = 10.4 Hz, $J$ = 5.4 Hz), 3.21 (1H, d, OH), 3.09 (1H, d, OH), 1.61 (9H, s), 1.60 (9H, s), 1.51 (3H, s), 1.33 (3H, s), 1.10 (9H, s).

$^{13}$C NMR (CDCl$_3$): δ 166.64, 163.45, 156.91, 135.71, 135.66, 133.14, 133.08, 130.04, 130.00, 127.97, 127.92, 115.92, 108.63, 81.71, 79.98, 78.27, 76.62, 69.95, 65.55, 65.30, 28.68, 28.61, 27.01, 26.90, 25.00 and 19.44.

HRMS [M +H]$^+$: calc. for. C$_{36}$H$_{52}$O$_7$N$_2$ Si, 653.3622; found 653.3604.

2.7.3. Synthesis of 2, 4-di-tert-butyldimethylsilyloxy-5-(4-(tert-butoxymethyl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxol-6-yl) pyrimidine (40)

![Chemical Structure](image)

To 39 (0.88 g, 1.34 mmol) in anhydrous THF, triphenylphosphine (1.06 g, 4.02 mmol) was added. To this solution at 0 °C, DIAD (0.79 ml, 4.02 mmol) was added dropwise and the solution slowly brought to rt and stirred for 65 hrs. The solvent was removed under vacuum to deliver a yellow oil. Purification by silica gel column chromatography using ethyl acetate and hexanes (15:85) gave 40 as a colorless oil (yield 60.7%).
$^1$H NMR (CDCl$_3$) : δ 8.34 (1H, s), 7.74 - 7.71 (4H, m), 7.43 - 7.37 (6H, m), 4.98 (1H, d, $J$ = 4.4 Hz), 4.68 (1H, dd, $J$ = 6.6, $J$ = 4.6 Hz), 4.59 (1H, dd, $J$ = 6.6 Hz, $J$ = 4.6 Hz), 4.15 (1H, q, $J$ = 4.4 Hz), 3.93 (1H, dd, $J$ = 11.2 Hz, $J$ = 3.9 Hz), 3.86 (1H, dd, $J$ = 11.2 Hz, $J$ = 4.6 Hz), 1.63 (18H, s), 1.60 (3H, s), 1.36 (3H, s), 1.08 (9H, s).

$^{13}$C NMR (CDCl$_3$) : δ 167.37, 163.92, 156.36, 135.75, 135.74, 133.35, 133.30, 129.85, 127.86, 127.83, 114.13, 114.11, 86.04, 84.52, 81.95, 81.66, 80.65, 80.11, 64.24, 28.67, 28.54, 27.78, 26.98, 25.79 and 19.34.

HRMS [M +H]$^+$ : calc. for. C$_{36}$H$_{50}$O$_6$N$_2$ Si, 635.3516; found 635.3496

2.7.4. Synthesis of (4-(2,4-di-tert-butoxypyrimidin-5-yl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxol-6-yl)methanol (41)

To a solution of 40 (0.51 g, 0.80 mmol), in dry THF, TBAF (1.20 ml, 1.20 mmol, 1.0 M solution in THF) was added dropwise at 4 ºC. The reaction mixture was slowly brought to rt and stirred for 1 hr. The solvent was removed in vacuum to deliver the crude product, which was immediately purified by silica gel column chromatography using ethyl acetate and hexanes (30:70) to give 41 as a white solid (yield 85.4%).
\(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.16 (1H, s), 4.81 (1H, d, \(J = 4.2\) Hz), 4.76 (1H, m), 4.73 (1H, m), 4.04 (1H, m), 3.89 (1H, dd, \(J = 11.8\) Hz, \(J = 3.1\) Hz), 3.76 (1H, m), 2.36 (1H, s, OH), 1.64 (9H, s), 1.59 (9H, s), 1.58 (3H, s), 1.34 (3H, s).

\(^{13}\)C NMR (CDCl\(_3\)): 167.73, 164.14, 157.57, 114.68, 112.61, 84.69, 84.23, 82.59, 81.95, 81.09, 80.43, 62.54, 28.62, 28.38, 27.58 and 25.56.

HRMS [M +Na]\(^+\): calc. for. C\(_{20}\)H\(_{32}\)O\(_6\)N\(_2\), 419.2158; found 419.2184

2.7.5. Synthesis of 6-(2, 4-di-tert-butoxypyrimidin-5-yl)-tetrahydro-2,2-dimethylfuro[3,4-d][1,3]dioxole-4-carbaldehyde (42)

![Diagram of compound 42]

To a solution of the Dess-Martin Reagent (680 mg, 1.6 mmol) in 16 ml of anhydrous CH\(_2\)Cl\(_2\) was added compound 41 (420 mg, 1.06 mmol) in 10 ml of anhydrous CH\(_2\)Cl\(_2\). The solution was stirred at room temperature for 24 hours and the solvent was evaporated to give the crude product. The residue was dissolved in ethyl acetate and stirred for 30 min and then filtered to remove excess Dess-Martin Reagent. It was purified by silica gel column chromatography using ethyl acetate: hexanes (1:1) to deliver pure product (yield 50%).

\(^1\)H NMR (CDCl\(_3\)): \(\delta\) 9.72 (1H, s), 8.16 (1H, s), 5.02 (1H, d, \(J = 2.9\) Hz), 4.94 (1H, m), 4.86 (1H, m), 4.42 (1H, d, \(J = 3.9\) Hz), 1.62 (9H, s), 1.60 (9H, s), 1.60 (3H, s), 1.37 (3H, s).
$^{13}$C NMR (CDCl$_3$): 200.43, 167.76, 164.47, 114.69, 112.59, 89.28, 84.44, 83.69, 83.53, 83.20, 82.39, 82.22, 80.83, 28.87, 28.68, 28.48, 27.54, 25.66, and 25.50

HRMS [M +OCH$_3$+Na]$^+$: calc. for. C$_{21}$H$_{34}$O$_7$N$_2$, 449.2246; found. 449.2246

2.7.6. Synthesis of 2-tert-butyldimethylsilyloxy-2-(4-(2, 4-di-tert-butoxypyrimidin-5-yl)-tetrahydro-2, 2-dimethylfuro [3,4- d][1,3]dioxol-6-yl)acetonitrile (43)

Compound 42 (160 mg, 0.41 mmol) was coevaporated with anhydrous dichloromethane (2 $\times$ 10 ml) and dissolved in anhydrous THF. To this solution, 18-Crown-6 (2.2 mg, 0.0082 mmol) and KCN (2 mg, 0.0287 mmol) were added and the solution stirred for 15 min at rt. To this was added TBDMSCN (145 mg, 1.02 mmol). The solution stirred for 4 hrs and was then quenched by the addition of saturated sodium chloride. The product was then extracted with ethyl acetate (3 $\times$ 10 ml) and the combined organic extracts dried over anhydrous sodium sulfate and the solvent removed under vacuum. Purification by flash column chromatography gave a mixture of diastereomers in a 4:5 ratio (NMR). (yield 41%).

$^1$H NMR (CDCl$_3$): $\delta$ 8.29 (1H, s), 8.21 (1H, s), 5.01 (1H, d, J = 4.6 Hz), 4.97 (1H, d, J = 4.6 Hz), 4.75 (2H, m), 4.67 (1H, m), 4.65 (1H, m), 4.63 (1H, m), 4.60 (1H, dd, J = 6.6 Hz, J = 4.6 Hz), 4.15 (2H, m), 1.64 (18H, s), 1.61 (18H, s), 1.60 (6H, s), 1.59 (3H,s), 1.37 (6H, s), 0.92 (9H, s), 0.91 (9H, s), 0.24 (3H, s), 0.22 (3H, s), 0.19 (3H, s), 0.18 (3H, s).
$^{13}$C NMR (CDCl$_3$): 167.43, 167.41, 164.15, 164.10, 156.69, 156.51, 118.16, 117.80, 115.06, 114.73, 112.98, 112.92, 85.69, 85.49, 84.79, 84.32, 82.42, 82.39, 81.58, 81.21, 81.15, 80.53, 80.46, 63.48, 62.98, 28.75, 28.59, 27.82, 27.80, 25.81, 25.79, 25.74, 25.68, 18.34, 1.25, -4.86, -4.95, -5.18.

HRMS [M+H]$^+$: calc. for C$_{27}$H$_{45}$O$_6$N$_3$Si, 536.3156; found. 536.3141
Chapter 3

Results and Discussions

3.1. Design and synthesis of C5'-pivaloyluridine

Reactive oxygen species, generated by endogenous and exogenous sources play a vital role in damaging biological molecules. These reactive oxygen species produce radicals through the abstraction of one or more hydrogen atoms from DNA and/or RNA. It is necessary to understand radical initiated damage at various positions on the nucleobase or sugar of nucleic acids, because studies show that oxidative damage caused by radical generation, plays a significant role in the pathophysiology of inflammation, cancer and neurodegenerative diseases.\(^8,32-34\) Much research focuses on understanding these mechanisms through independent generation of radicals at a specific position in nucleosides and nucleotides (Scheme 1-1 and 3-1).\(^{15,35}\)

Synthesizing suitable radical precursors is the best approach to make these studies possible, because these radical precursors undergo homolytic photocleavage to generate a radical at a specific position. That is why, many research groups focus on synthesizing radical precursors with photolabile groups that can generate radicals at a desired position. Hence,
the same approach has been employed for this study utilizing a \( t \)-butylketone (pivaloyl group) as a photolabile moiety.

The goal of our research group is to study and understand mechanisms involved in the damage caused by sugar radicals in DNA and RNA. The objective of the present study is to develop the tool, utilizing the synthetic approaches, required for understanding oxidative damage to RNA at a molecular level. We are studying the damage caused due to the generation of radicals at the C5' position of the ribose sugar in nucleotides.

\[
\text{Scheme 3-1: Generation and fate of the 2'-deoxyuridin-1'-yl radicals}
\]

Few studies have been shown in the literature that focus on C5'-sugar radicals in nucleic acids. Furthermore, no studies have been reported on C5'-ribose radicals in ribonucleotides. The position of the C5'-ribose sugar is a very important site to investigate radical mediated
damage to RNA. Studies on C5' oxidation in DNA did not gain much attention due to the lack of abasic site formation (Scheme 3-1).\textsuperscript{36} Most of the studies on C5' chemistry utilized agents such as Fe\textsuperscript{2+}-EDTA, metal complexes and antibiotics which are non-specific damaging agents.\textsuperscript{37} There is only one model study available for site specific generation of the C5' radical.\textsuperscript{20}

Therefore, it would be interesting to study the nature of the products obtained from the C5'-ribose radicals through independent generation. In order to carry out this study C5'-pivaloyluridine 27 was successfully synthesized and utilized for the generation of the C5' uridinylradical 28. Synthesis of C5'-pivaloyluridine began with compound 49 which was synthesized from uridine according to literature procedures (Scheme 3-2).\textsuperscript{38,39}
It is important to mention that compound 49 easily undergoes hydration to give a C5' hydrate. Hence, the reaction was performed under strictly anhydrous conditions. Exposure of the product to an aqueous work up was avoided due to the known formation of the C5'-hydrate under these conditions. Formation of the uridine C5'-hydrate was determined by ESI-MS. Using other oxidizing reagents like TEMPO and Swern oxidation did not deliver the desired product due to solubility issues related to the reactant. We observed that reaction with the Dess-Martin Reagent was cleaner than with other reagents, however contamination with the DM reagent was observed in the final product. This did not however

**Scheme 3-2**: Synthesis of C5'-pivaloyluridine (27)
interfere with the formation of the desired cyanohydrin in the next synthetic step. Therefore, the crude product was utilized for this step.

Compound 49 was converted to the protected cyanohydrin (34) by treatment with catalytic amounts of KCN and 18-Crown-6 followed by 2.5 equivalents of t-butyldimethylsilyl cyanide (Scheme 3-2). Compound 34 was obtained as two isomers in a 4:5 ratio under these conditions. These two isomers were collected together and analyzed by NMR. In proton NMR, two doublets were identified at 4.75 ppm and 4.71 ppm, corresponding to the H-5' hydrogen atom of each isomer. The H-6 proton appeared as two doublets at 7.44 and 7.28 ppm corresponding to two protons. Additionally, a signal which corresponds to a t-butyl group was observed at both 0.91 and 0.87 ppm, indicating the presence of two isomers. Signals for methyl groups were observed between 0.22 to 0.10 ppm for both isomers (Figure A-1).

\[^{13}C\text{ NMR showed the presence of a signal corresponding to a cyano group at 118.6 and 117.8 ppm indicating both isomers (Figure A-2). This evidence sufficiently supports the formation of the desired product (34). Furthermore, high resolution mass spectra showed the molecular ion peak (M +Na) at m/z 446.1725 (calculated 446.1723), which further confirms product formation (Figure A-3). After successfully obtaining compound 34, it was reacted with t-butyllithium (t-BuLi) at -78 °C to install a t-butylimino group at the C5'\]
position (Scheme 3-2). This compound was very unstable, and was consequently subjected to hydrolysis without further purification. Hydrolysis of 35 by the addition of THF/ H$_2$O/ 2N HCl (40:20:1) delivered fully protected C5'-pivaloyl uridine 36 (Scheme 3-2).

Synthesis of 36 was also achieved by the addition of 1N HCl (pH 2.0) to the reaction mixture without isolating imine intermediate 35. However, we found that isolation of 35 followed by hydrolysis results in a higher yield of 36. Two diastereomers were obtained in 1.5:1 ratio as determined by NMR. This mixture of diastereomers was carried to the next step without separation. $^1$H NMR showed the presence of two doublets at 4.93 and 4.91 ppm which correspond to the H-5' hydrogen atom of two isomers (Figure A-4). The H-5' hydrogen atom for compound 36 shifted downfield when compared with compound 34. This provided an indication of conversion of the cyano group to the $\tau$-butylketone group.

Compound 36 was also characterized by $^{13}$C NMR, which clearly showed the presence of the ketone group. Peaks at 213.39 and 211.26 ppm were observed corresponding to ketone groups from each isomer (Figure A-5). This evidence along with the data obtained from HRMS confirmed the formation of product 36 (Figure A-6).

Compound 36 was then treated with tetra-$n$-butylammonium fluoride to deprotect the C5'-hydroxyl group. Resulting product 121 was obtained in a 1.5:1 ratio as two diastereomers.
Disappearance of signals for the TBDMS groups in proton and $^{13}$C NMR confirmed product formation (Figure A-7 and A-8). Formation of 121 was further confirmed by HRMS analysis (Figure A-9). Finally, compound 121 was refluxed with 25% acetic acid for 1 hour to remove the isopropylidene protecting groups. This reaction gave fully deprotected C5'-pivaloyluridine, 27 (Scheme 3-2) as a mixture of two isomers in a 2:1 ratio. Formation of this compound was confirmed by NMR and HRMS (Figure A-10 and Figure A-12). Proton NMR showed the disappearance of the isopropylidene group between 1.60 to 1.30 ppm. This was further confirmed by $^{13}$C NMR (Figure A-11).

After successful synthesis, the X-ray crystal structure of 27 was solved (Figure 3-1). Crystals suitable for X-ray analysis were obtained from a saturated solution of methanol. The isomer which was crystallized under these conditions was determined to have the $R$ configuration at C5'.
The ribose ring was found to possess a C2' \textit{endo} sugar pucker. This is in contrast with the uridine crystal structure sugar confirmation. A crystal structure of uridine showed a C3' \textit{endo} sugar conformation (Figure 3-2).\textsuperscript{40,41} The differences in the orientation of the sugar in 27 compared to uridine could be attributed to the presence of the exocyclic \textit{t}-butylketone group at the C5'-position.

\textbf{Figure 3-1}: X-ray crystal structure for 27 in two orientations
3.2. Investigation of the suitability of 27 for use as a precursor of the C5'-uridinyl radical

Independent generation of radicals through the synthesis of suitable radical precursors is the most useful method to study radical derived product formation. Radical precursors smoothly undergo photochemical cleavage under suitable photolytic conditions and deliver the radical of interest. These precursors generate radicals in higher yields with limited side reactions. This strategy has been utilized by several research groups to study damage products in nucleosides and nucleotides.\textsuperscript{42-45}

This method provides a straightforward approach for the study of unknown damage products obtained from the generation of radicals at a specific position. In addition, site specific generation of radicals provides information on what kind of damage products can be expected from a radical at a particular site.
This approach prevents the formation of undesirable products which otherwise make the characterization of these unknown products very difficult. Predicted products can be synthesized and used as standards for comparative studies. Hence this has been proven to be an extremely useful approach to investigate the mechanism for formation of damage products in biological systems. The tert-butylketone group has been established as a suitable photolabile moiety to generate radicals at a specific site in nucleosides and nucleotides. Several examples of modified nucleosides utilizing this group are shown in Figure 3-3.

Radical precursors using this group have been synthesized at different positions on nucleosides. These modified nucleosides were also incorporated into oligonucleotides to study the radical induced products under more biologically relevant conditions. In addition
to the tert-butylketone moiety, other groups such as methyl ketones (54) and phenyl selenides (55, 56) have been utilized for selective generation of radicals (Figure 3-4). These groups were however, found to be less efficient compared to tert-butylketones. Tert-butylketones smoothly undergo Norrish type I photocleavage to generate radicals at a desired position without forming by-products which interfere with studies. On the other hand, methyl ketones can undergo Norrish type II reactions which lead to the formation of undesirable by-products. Similarly, phenyl selenides showed the formation of multiple photoproducts from different pathways which lowers the chemical yield of the desired radical. Therefore tert-butylketones are more commonly used for the synthesis of radical precursors.

Through careful comparison of these and other precursors we decided that the tert-butylketone group would likely be most efficient as a radical precursor in our system to study the damage products obtained from the generation of the C5'-uridinyl radical. Photolysis of 27 was carried out in the presence and absence of a hydrogen atom donor. When an H-donor was used tri-n-butyltin hydride or glutathione were employed. In addition, photolyses was performed under anaerobic and aerobic conditions.

**Scheme 3-3:** Photochemical generation and fate of the C5'-uridinyl radical
After photolysis of 27, the crude photolysates were injected onto an HPLC column and fractions were collected and analyzed by mass spectrometry.

3.2.1. Photochemical generation of the C5' uridinylradical in the presence of tri-n-butyltin hydride (nBu₃SnH)

To study the efficiency of the tert-butylketone moiety as a radical precursor, it was necessary to trap the radical generated at the C5' position of uridine using a hydrogen atom donor.

To facilitate the identity of the products a hydrogen atom donor which produces the radical reduction product was chosen. Tri-n-butyltin hydride is a strong hydrogen atom donor which is expected to minimize all other degradation pathways and lead to the formation of the reduction product exclusively. Initial photolysis experiments were carried out in the presence of excess tri-n-butyltin hydride (500 equivalents).
Photolyses was performed for 15, 30, 45 and 60 min to estimate the time required for the complete photochemical conversion of 27 into radical derived products. It was noticed that radical precursor 27 was completely consumed in 60 min, leading to the formation of the product (Figure 3-5). Hence, 60 min was applied to the rest of the photolysis experiments. These photolysis experiments were carried out under anaerobic conditions using an CH$_3$CN: H$_2$O (1:1) solvent system. An intense peak was observed at ~ 4.86 min. This retention time did not match the retention time of the expected product, uridine (26). Under these conditions formation of uridine (26) was not detected. The only product that formed was uracil 57 (Scheme 3-4). Formation of this compound was identified by spiking the photolysate of 27 with commercially available uracil.

Furthermore, MALDI-TOF analysis of the fraction which eluted at ~ 4.86 min confirmed the formation of 57 (Figure A-18). This experiment was repeated with higher
concentrations of C5' radical precursor (0.37 mM) as compared to our initial experiments to study the role of precursor concentration on the distribution of products.

![Figure 3-6](image)

**Figure 3-6**: Chromatogram for 27 (0.37 mM) after the photolysis 500 equiv. of nBu$_3$SnH in 1:1 CH$_3$CN/ H$_2$O

This photolysis was also carried out for 15, 30, 45 and 60 min, which also resulted in the formation of base elimination product 57 (**Figure 3-6**). Increasing the concentration of tri-$n$-butyltin hydride (1000 eq) did not suppress the formation of uracil (57). Changing the photolysis parameters did not result in the formation of the reduction product. The chromatogram for the formation of 57 in the presence of tri-$n$-butyltin hydride is shown below (**Figure 3-7**).

![Figure 3-7](image)

**Figure 3-7**: Chromatogram for the formation of uracil, 57
Based on the results obtained, it was obvious that increasing the concentration of radical precursor and/or increasing the concentration of tri-\(n\)-butyltin hydride did not provide the expected reduction product. Tri-\(n\)-butyltin hydride is not very soluble in 1:1 CH\(_3\)CN:H\(_2\)O, which may hinder its ability to reduce the radical. Therefore, photolysis of 27 was carried out in CH\(_3\)CN alone with a minimal amount of H\(_2\)O (~6%). After photolysis, the crude photolysate was evaporated to remove excess acetonitrile and then injected onto an HPLC column. Formation of the reduction product was not observed under these conditions either. Based on these results, it was concluded tri-\(n\)-butyltin hydride was not suitable to produce the radical reduction product. Due to the issues related to solubility of the precursor and tri-\(n\)-butyltin hydride we chose to utilize fully protected C5'-uridylnylradical precursor 36 for photolysis which has greater solubility in acetonitrile than 27. Photolysis was carried out in 2:1 v/v CH\(_3\)CN: H\(_2\)O in the presence of 1000 equivalents of tri-\(n\)-butyltin hydride (Scheme 3-5). The crude photolysate was concentrated to remove excess acetonitrile and injected onto an HPLC column. Products formed were compared with independently obtained standards. This experiment gave interesting results, with the formation of reduction product, 37. The product identity

**Scheme 3-5:** Photochemical generation and fate of the C5'-uridylnylradical in 36 in the presence of 1000 equiv. \(n\)Bu\(_3\)SnH
was confirmed through comparison with the independently synthesized standard (Figure A-19). MALDI-TOF of the photolysate confirmed the presence of the reduction product (Figure A-20). However, the reduction product was not the major product. Along with 37 a strong peak for uracil (57) was observed. Several other unidentified peaks were also observed.

It is difficult to postulate the mechanism of formation of uracil (57) through the generation of the C5'-uridinylradical. If we assume 57 was obtained from the C5' radical, it can be hypothesized that generation of this reactive intermediate leads to products which result in base elimination. This could explain why the formation of the reduction product was not observed when C5'- pivaloyluridine was subjected to photolysis in the presence of tri-n-butyltin hydride. In the case of 2', 3', 5' protected C5'- pivaloyluridine, it can be postulated that the presence of protecting groups plays a role in the hindrance of the formation of the products that lead to base elimination. It can be postulated that protecting groups stabilize the C5'radical allowing enough time for abstraction of the hydrogen atom from the corresponding donor or the absence of protecting groups on the nucleoside, makes it is much easier for the compound to undergo base elimination. Bulky substituents on the nucleoside are known to influence product distribution.49 Chatgilialoglu’s group observed a change in the ratio of stereoisomers depending on the presence/ absence of substituents on the C5', 2' hydroxyl groups. According to the study, the C5'-deoxy-adenosylradical undergoes cyclization to form (5'S, 8R)-cyclodeoxyadenosine and (5'R, 8R)-cyclodeoxyadenosine. The ratio of these stereoisomers was strongly influenced by the presence of protecting groups and the polarity of the solvent.
A 90:10 ratio of 5'S:5'R was observed when the 2' and 5'-hydroxyl groups are protected with TBDMS, and a 55:45 ratio was observed when no protecting groups are present (Figure 3-8). The differences in this ratio was explained in terms of steric hindrance introduced by the TBDMS group, which causes the sugar to favor a C5'-endo confirmation. In the absence of the TBDMS group, cyclization is nonstereoselective. Though this cannot be applied directly to our system, it can be postulated that the presence of the TBDMS group affects the confirmation of the sugar in the precursor and subsequently the fate of the radical. In the present study, it can be concluded that sterics influence the kind of products formed.

However, no conclusions should be made at this stage without completely exploring the behavior of the radical. To gain further insight into the nature of the radical precursor and understanding the C5'-radical chemistry, radical precursor 27 was subjected to photolysis in the presence of other hydrogen atom donors.
3.2.2 Photochemical generation of the C5'-uridinyl radical in the presence of glutathione (GSH)

Photolysis of 27 was carried out in the presence of the physiologically relevant hydrogen atom donor, glutathione (Scheme 3-6).

![Scheme 3-6](image)

**Figure 3-9:** Chromatogram for the photolysate of 27 in the presence of 1000 equiv. of glutathione. **Purple:** radical precursor 27; **Blue:** uridine std + GSH; **Red:** photolysate; **Green:** photolysate spiked with uridine std
Initially, photolysis of 27 (0.10 mM) was carried out at a higher concentration of glutathione (100 mM) using water as solvent. The sample was photolysed for one hour under anaerobic conditions. Formation of the reduction product (26) was observed under these conditions and its identity was confirmed by spiking the photolysate with a uridine standard. This confirms that 27 successfully generates the C5'-uridinyl radical which then abstracts a hydrogen atom from glutathione to give uridine 26 (Figure 3-9). However, it is not clear why uridine (26) formed in the presence of glutathione and not in the presence of other hydrogen atom donors. This indicates the necessity to further explore the behavior of the radical precursor and determine efficient conditions to generate the reduction product.

**Figure 3-9**, shows peaks for glutathione appearing at ~ 4.5 min which interfere with the detection of uracil, which elutes at ~ 4.7 min. This problem was eliminated by using lower concentrations of glutathione. Moreover, lower concentrations of glutathione are more relevant to the simulation of the physiological conditions for the study of interest. Studies have shown that intracellular levels of glutathione in mammalian cells are in the range of 0.5-10 mM. Experiments were therefore designed to determine the impact of different concentrations of glutathione in the low mM range (0-32 mM) on the products formed through generation of the C5'-uridinyl radical.

### 3.2.2.1. Photolysis of 27 using different concentrations of glutathione at pH 7.0

Photolysis of 27 (0.375 mM) was performed using glutathione concentrations ranging from 0 to 32 mM in 10 mM phosphate buffer under anaerobic conditions.
Formation of uracil (57) and uridine (26) were observed under these conditions. Representative examples are shown for each concentration of glutathione in the appendix (Figure A-21 to A-26). Formation of 26 and 57 was confirmed by spiking with standards (Figure A-27). MALDI-TOF analysis confirmed the formation of 26 and 57 (Figure A-28 and A-29). Mass balances for the formation of these two products were calculated based on calibration curves (Table 3.1). Formation of 57 was observed in the absence and presence of glutathione. The yield of 57 slowly increased with increasing concentrations of glutathione up to 8 mM. Further increases in the concentration of glutathione to 32 mM reduced the yield of 57. The maximum yield of 57 (47.8%) was observed with 8 mM glutathione.

Table 3.1: Mass balances of products from photolysis of 27 in the presence of GSH at pH 7.0

<table>
<thead>
<tr>
<th>GSH(mM)</th>
<th>Uridine(%) 26</th>
<th>Uracil(%) 57</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N.D ± 0.00</td>
<td>33.9 ± 0.83</td>
<td>33.9 ± 0.83</td>
</tr>
<tr>
<td>2</td>
<td>7.12 ± 0.12</td>
<td>43.7 ± 4.98</td>
<td>50.8 ± 5.10</td>
</tr>
<tr>
<td>4</td>
<td>9.44 ± 0.02</td>
<td>44.3 ± 3.26</td>
<td>53.7 ± 3.28</td>
</tr>
<tr>
<td>8</td>
<td>7.30 ± 1.45</td>
<td>47.8 ± 2.86</td>
<td>55.1 ± 4.31</td>
</tr>
<tr>
<td>16</td>
<td>7.79 ± 2.96</td>
<td>43.1 ± 3.75</td>
<td>50.9 ± 6.71</td>
</tr>
<tr>
<td>24</td>
<td>10.5 ± 0.87</td>
<td>42.3 ± 5.51</td>
<td>52.8 ± 6.38</td>
</tr>
<tr>
<td>32</td>
<td>15.4 ± 0.55</td>
<td>43.3 ± 3.21</td>
<td>58.7 ± 3.76</td>
</tr>
</tbody>
</table>
On the other hand, there was a slow but steady increase in the yield of 26 (Figure 3-10).

The maximum amount of 26 (~15%) was formed with 32 mM glutathione. There was no

**Figure 3-10:** Formation uridine (26) and uracil (57) upon photolysis of 27 in the presence of different concentrations of GSH at pH 7.0
drastic increase in the yield of products with glutathione concentrations from 2 to 32 mM. These results were unexpected as it was assumed that only the formation of 26 would be observed. Surprisingly, the major product was base elimination product 57. With this result we decided to investigate the role of pH in product distribution. The basis for this assumption is that when photolysis was performed with 1000 equivalents of glutathione in water, the major product obtained was the reduction product, 26. Under these conditions the sample pH was not adjusted to 7.0. It is known that the addition of glutathione to aqueous solution lowers the pH. Hence, we believed that pH plays a crucial role in the reducing abilities of glutathione in this system.

3.2.2.2. Photolysis of 27 using different concentrations of glutathione at pH 3.5

<table>
<thead>
<tr>
<th>GSH(mM)</th>
<th>Uridine(%) 26</th>
<th>Uracil(%) 57</th>
<th>Total(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N.D ± 0.00</td>
<td>13.6 ± 1.15</td>
<td>13.6 ± 1.15</td>
</tr>
<tr>
<td>2</td>
<td>30.3 ± 0.75</td>
<td>10.4 ± 0.45</td>
<td>40.7 ± 1.20</td>
</tr>
<tr>
<td>4</td>
<td>45.7 ± 2.10</td>
<td>11.2 ± 1.09</td>
<td>56.9 ± 3.19</td>
</tr>
<tr>
<td>8</td>
<td>54.6 ± 0.45</td>
<td>11.2 ± 0.17</td>
<td>65.8 ± 0.62</td>
</tr>
<tr>
<td>16</td>
<td>66.9 ± 2.39</td>
<td>10.7 ± 0.17</td>
<td>77.6 ± 2.56</td>
</tr>
<tr>
<td>24</td>
<td>70.0 ± 0.72</td>
<td>8.38 ± 1.52</td>
<td>78.3 ± 2.24</td>
</tr>
<tr>
<td>32</td>
<td>75.9 ± 1.82</td>
<td>8.55 ± 1.26</td>
<td>84.5 ± 3.08</td>
</tr>
</tbody>
</table>

Table 3-2: Mass balances of products from photolysis of 27 in the presence of GSH at pH 3.5
The same experimental conditions were employed as above with the exception that the pH was adjusted to 3.5. The reason to choose this pH, is that we wanted to select a suitable pH that can be used for all the concentration of GSH. A 32 mM solution of GSH in water has a pH of 2.8 which was found to be too acidic for our experimental setup.

Therefore, we chose a slightly higher pH which can be employed for all the concentrations of GSH under study without forming a significant amount of degradation product. This pH was kept constant for all the concentrations of glutathione. Representative chromatograms for each concentration of glutathione are shown in the appendix (Figure A-30 to A-36). Under these conditions again formation of 26 and 57 were observed. Yields of the two major products uridine 26 and uracil 57 were calculated (Table 3-2). Here the major product observed was reduction product 26. This study confirmed that pH plays a major role in product distribution. It was observed that there was a gradual increase in the yield of uridine (26) as the concentration of glutathione increased. On the other hand, there was a slow decrease in the yield of uracil with increasing glutathione concentration (Figure 3-11).
The maximum amount of uridine (75.9%) was formed with 32 mM glutathione and the maximum amount of uracil formation was observed with 0 mM glutathione. This study clearly shows that acidic conditions are required for the formation of reduction product,
Product 57 however remained constant at all concentrations of glutathione, which indicates that the formation of uracil is independent of glutathione concentration.

**Scheme 3-7:** Photolysis of 27 in presence of glutathione

This suggest that base elimination is the main pathway of degradation at physiological pH. This also indicates that glutathione can act as a potential hydrogen atom donor under acidic conditions, but is much less efficient in a neutral environment. These results show that glutathione concentration and pH play a dominant role in determining the yields of the products formed via the C5'-uridinylradical (**Scheme 3-7**). Formation of products 26 and 57 were confirmed by spiking the photolysate of 27 with uridine and uracil standards. Furthermore, mass spectral analysis of the HPLC fractions confirmed product formation. This study also indicates that glutathione acts as a strong hydrogen atom source under acidic conditions. It also showed that the pH of the system was the dominant factor influencing the formation of reduction product, 26. An explanation for the observed outcome lies in the activity of glutathione under different conditions. Based on a study by
Simic’s group, glutathione exhibits different radical reducing mechanisms depending on the pH of the system. It can reduce free radicals through hydrogen atom donation or electron transfer. In the case of carbon centered radicals, hydrogen atom transfer is the common phenomenon. In order to deliver the hydrogen atom, GSH has to be in the reduced form. At lower pH GSH is predominantly in its reduced form, allowing it under photolytic conditions to deliver a hydrogen atom to the C5'-uridylnylradical. When the pH goes from neutral to basic, GSH is mostly in its GS⁻ form and unable to deliver a H-atom. Therefore, we can say that GSH is a stronger hydrogen atom donor at lower pH. This explains the importance of pH in our study.

3.3. Photochemical generation of the C5'-uridylnylradical using water as solvent

To investigate the role of solvent on the yields of 26 and 57, photolysis of 27 was carried out in water (~6% CH₃CN) using different concentrations of glutathione as hydrogen atom donor without adjusting the pH. Under these conditions formation of 26 and 57 was observed. (Figure A-37 and A-38). The yield of 26 increased with increasing glutathione concentration and was compatible to those obtained from photolysis in buffer at lower pH (Figure 3-12). Uracil was obtained under all experimental conditions, indicating that formation of this compound could be through the generation of a radical cation at C1' or C2'. Interestingly, photolysis of 27 in water gave a better chromatogram compared to the samples obtained in buffer (Figure A-41). When buffer was used as solvent here, encountered issues with lack of base line separation of peaks.
However, photolysis of 27 in buffer is very important to understand the role of pH on product distribution. Therefore, these results gave an indication that generation of the C5'-
radicals in uridine might lead to base elimination under physiological conditions if not repaired by the body’s defense systems. However, making such assumptions is beyond the scope of the present work.

Overall, photolysis of 27 successfully delivered the desired C5' radical which was trapped by glutathione. These results were reproduced using both water and phosphate buffer. The study revealed that the pH of the solution and glutathione concentration are the two main factors that affect the yields of the photolysis products. Base elimination was observed in the presence and absence of glutathione. Possible mechanisms for the formation of reduction product and base elimination are shown below.

**Formation of reduction product (26)**

![Chemical Diagram of Formation of Reduction Product]

**Scheme 3-8: Mechanism for the formation of reduction product 26**

**Base elimination**

C5'-oxidation in DNA by antibiotic antitumor agents bleomycin, neocarzinostatin and calicheamicin follows two pathways (Scheme 3-9), one pathway gives strand break products containing 3'-formylphosphate (61) ends and 5'-[(2-phosphoryl-1,4-dioxo-2-butane)] (62) fragments, while the second pathway leads to 5'-nucleoside-5'-aldehyde (63) and 3'-phosphate (64). Another model study by Chatgilialoglu’s group revealed
through selective generation of the C5'-radical utilizing purine bases leads to the formation of 5',8-cyclo-2'-deoxynucleoside products through intramolecular attack on the C8-N7 double bond of purine bases.\textsuperscript{10,20,54}

\textbf{Scheme 3-9:} Products derived from C5'-oxidation in DNA

Studies shows that the reactivity of the ribose sugar is different from 2-deoxyribose.\textsuperscript{17} This assumption is also supported by the work of Giese’s group on adenosine.\textsuperscript{55} In this study the C8-adenosine radical was generated and the products obtained from this radical were shown (Scheme 3-10). In one pathway, 5',8-cycloadenosine (68) was observed through the abstraction of C5'-hydrogen atom. Studies on 2'-deoxyadenosine also showed the formation of 5',8-cyclo-2'-deoxyadenosine upon the generation of the C8-radical.\textsuperscript{50} Formation of the cyclized product was the major product under anaerobic conditions.

In this study a new pathway was also observed as postulated in Scheme 3-10 which may involve the intramolecular abstraction of the C2'-hydrogen which has never been observed in DNA studies before. This indicates that the reactivity of ribose and 2'-deoxyribose are
different. In addition, radical transfer to the C2'-position lead to base elimination. Radical translocation to the C2'-position is possible because of the smaller bond distance between the C8-radical and the C2'-hydrogen (2.70 Å), although the activation energy is 9.6 kcal/mol.

Once the C2'-radical is generated it undergoes heterolytic β C-N bond cleavage to the give base elimination product. From these studies we observe that there is a compromise between activation energy for the intramolecular hydrogen atom transfer and bond distance.\textsuperscript{55}

Scheme 3-10: Fate of C8-adenosylradical\textsuperscript{55}
In our system, cyclization between nucleobase and sugar is less likely because of the longer bond distances between the C5'-C6 of the nucleoside due to the smaller size of the base (pyrimidine base). However, there is a possibility of radical transfer to the C2'-position as well. The reason for this assumption lies in the bond distance between C5' and the H-2' atom. This distance was observed to be 3.14 Å (based on x-ray crystal structure analysis). This is shorter than the distance of the C8-radical to the C5'-postion (3.84 Å) in the above mentioned system. Although the activation energy was not measured, this assumption is still reasonable if we consider the fact that the C2'-OH group stabilizes the radical through the inductive effect, in addition to the fact that the C5'-radical is a secondary radical which is less stable than the tertiary C2'-radical. Based on the above assumption, a proposed mechanism for base elimination is shown in **Scheme 3-11**.
An alternative approach for the formation of base elimination could be through the generation of a pseudo C4'-radical as the mechanism proposed by Chatgilialoglu’s group. According to this study, a pseudo C4'-radical is generated under aerobic conditions through an oxy radical intermediate. However, formation of this oxy radical is not understood. This oxy radical undergoes β-fragmentation to give a C4'-radical, which is responsible for the fragmentation products (Scheme 3-12). In our study we were unable to identify any sugar fragments, therefore it is difficult to say what could be the most possible mechanism for base elimination.

Scheme 3-12: Proposed mechanism for the base elimination under aerobic conditions
3.4. Photochemical generation of the C5'-uridinylradical under aerobic conditions

Photochemical generation of the C5'-uridinyl radical under aerobic conditions in the presence of 6 mM glutathione produced 26 and 57 as major products (Figure 3-13). These experiments were carried out in water. In addition, photolysis of 27 under aerobic conditions in the absence of a hydrogen atom donor showed the formation of only base elimination product 57 (Figure 3-14). Product formation was confirmed by comparison with standards.

![Chromatogram](image)

**Figure 3-13**: Chromatogram for the photolysis of 27 under aerobic conditions with 6 mM GSH. Blue: Standards; Brown: Photolysate of 27
Analysis of the crude photolysate using MALDI-TOF (Figure A-39 and A-40) showed the formation of new compounds which were not observed on the column. Photolysis of 27 under aerobic conditions lead to the formation of C5' aldehyde (74) and its associated C5' hydrate (75). These products could come from superoxide release from 72 which degrades to compound 74. The C5' aldehyde under aqueous conditions exist partially in the form of the hydrate (75) (Scheme 3-13). The alternate pathway to form 75 by the release of superoxide from 72 to deliver a C5'-cation, which upon addition of water give 75.
3.5. Synthesis of a C5'-pivaloylpseudouridine

The synthesis of C5'-pivaloyluridine was successfully achieved and this radical precursor was subjected to photolytic cleavage for the generation of the C5'-uridinyl radical. Products obtained from C5'-uridinyl radicals were analyzed by HPLC and mass spectrometric methods. Possible mechanisms for the formation of these radical derived products were proposed. The nucleobase elimination product, uracil was observed under all experimental conditions. It was proposed that the pH of the solution plays a dominant role in product distribution. Depending upon the concentration of glutathione, the ratios of uracil and uridine changed. Under aerobic conditions, in addition to uracil, formation of the C5'-aldehyde and C5'-hydrate were also observed.

As we know, pseudouridine is a C-glycoside and uridine is an N-glycoside. It would be interesting to observe the influence of the presence of a C-C bond on the product
distribution upon the photochemical generation of the C5'-radical. The strategy applied to the generation of the C5'-uridinyl radicals will therefore be extended to C5'-pseudouridinyl radicals.

3.5.1. Synthesis of Pseudouridine

Various methods have been established for the synthesis of pseudouridine. It is important to introduce these methods to understand our approach for the synthesis of pseudouridine.

3.5.2. Known approaches for the synthesis of pseudouridine

The synthesis of pseudouridine was first reported by Shapiro et al.\textsuperscript{56} According to their procedure 2, 3, 5-tri-benzoyl-D-ribofuranosyl chloride was treated with lithiated pyrimidine to give the desired nucleoside (\textbf{Scheme 3-14}). In this reaction a 6-membered heterocyclic ring containing derivative was also observed as an undesired product. This was formed due to the protonation of the bridge oxygen during the hydrolysis of the protecting groups. The reported total yield for four isomers was found to be 3.5\%. In this synthesis, the key step is the coupling between lithiated base and protected sugar to maximize the yield and minimize the formation of the undesired 2, 4 dimethoxy uracil, which is obtained by the abstraction of a proton by the lithiated base.\textsuperscript{57}
From this study, we observed that the deprotection is an important step. Because strongly acidic conditions for deprotection lead to the formation of the undesired 6-membered hexose ring and lower overall yields of desired nucleoside. Another strategy for pseudouridine synthesis was developed by Brown’s group.\textsuperscript{57,58}

**Scheme 3-14:** Synthesis of pseudouridine by Shapiro et al.\textsuperscript{56}
Their synthetic strategy involved using a benzyl protected sugar instead of the benzoyl protection strategy reported in earlier work.\textsuperscript{56} Pyrimidine was protected with tert-butoxy groups which are more acid labile when compared to the methyl ethers reported in the study by Shapiro. Addition of the lithiated pyrimidine, which was formed by treatment of the protected base with \textit{n}-BuLi at -78 °C, resulted in the formation of D-\textit{altro} and D-\textit{allo} sugars in a 5:2 ratio, respectively. Cyclization of 84 using ethanolic HCl delivered α and β isomers of pseudouridine (85 and 86) respectively, in the same ratio (Scheme 3-15). This procedure was found to be superior to Shapiro’s method. Finally, the benzyl groups were cleaved by BCl\textsubscript{3} at low temperature to give pseudouridine in good yield (42%). They also investigated this strategy using 2,3-isopropyliene-D-ribose with lithiated di-tert-butoxypyrimidine. Under these conditions, an unusual product was observed, which was confirmed by spectral analysis (Scheme 3-16).
Though Brown’s protocol for pseudouridine synthesis encountered some problems with the formation of sugar derivatives upon removal of the benzyl groups, it is still a very good method for the synthesis of pseudouridine. Another method was developed by Chow’s group which was similar to Brown’s protocol (Scheme 3-17). In this study 2', 3'-isopropylidene-ribonolactone was employed instead of 2',3'-isopropylideneribose. The coupling reaction in this study gave a 1:8 mixture of α/β isomers which was higher than that obtained from Shapiro’s method. The C1' alcohol was reduced using BF₃·Et₂O/ Et₃SiH followed by deprotection in two steps.

Scheme 3-16: Mechanism for the formation of by-product
In our study we employed this method for pseudouridine synthesis. We however encountered issues with reduction of the C1' alcohol by BF₃.Et₂O/ Et₃SiH. The procedure indicated that the reaction should be performed at 4 °C. The reaction never went to completion at this temperature or at RT. Addition of more equivalents BF₃.Et₂O or Et₃SiH resulted in degradation. This drastically reduced the yield of the product. We were able to recover only ~5% of the β isomer. Therefore, we switched to a different route developed by Hanessian et al.⁶¹,⁶²

**Scheme 3-17:** Synthesis of pseudouridine by Chow et al.⁵⁹
In this strategy protection of the 2',3',5'-hydroxyl groups was performed in a single step, and the C1' hydroxyl group was reduced in the presence of L-selectride with ZnCl\textsubscript{2} to deliver D-\textit{altro} hexitol as a single isomer due to a \textit{Si} face attack (Scheme 3-18). In the absence of ZnCl\textsubscript{2} the major product was D-\textit{allo} hexitol. The absence of ZnCl\textsubscript{2} leads to a non-chelated intermediate as shown in Figure 3-15, which favors \textit{Re}-face attack at the carbonyl group. Cycloetherification under Mitsunobu conditions, followed by deprotection gave \textgreek{a} and \textgreek{b} isomers in high yields.
3.5.3. Synthesis of pseudouridine in the present study

For our study we adopted the method developed by Hanessian et al\textsuperscript{61} with some modifications. According to Hanessian’s protocol, the 2, 3, 5-hydroxyl groups of the ribonolactone were protected with 2, 2-dimethoxy propane in the presence of an acid catalyst at 60 °C. We encountered issues with this reaction that resulted in incomplete conversion to the product. Increasing reaction times and refluxing conditions did not improve the yields. This is the first step in the synthesis of pseudouridine, therefore low yields at this stage are not acceptable. Hence, we decided to optimize the synthesis by modifying the protection group strategy. During our previous synthesis of pseudouridine using Chow’s procedure, the 2',3'-hydroxyl groups of the ribose sugar were protected with an isopropylidene group and 5'-hydroxyl with a \textit{tert}-butyldiphenylsilyl (t-BuPh\textsubscript{2}Si) group. Though it is a two-step synthesis, it gave very high yields of the desired products.

\textbf{Figure 3-15:} Pathway for stereoselective reduction of lactol\textsuperscript{61}
The use of the $t$-BuPh$_2$Si also provided a means to maintain a protected pseudouridine derivative. This group smoothly undergoes deprotection under mild conditions using TBAF, while all other protecting groups remain on the nucleoside.

Using this approach, synthesis of pseudouridine utilized compounds 110 and 92 which were synthesized using literature procedures.$^{58,61,63}$ Compound 110 was lithiated using $n$-BuLi at -78 °C and the resulting anion was added to a solution of 92 to deliver compound 38 (Scheme 3-19). This reaction resulted in the formation of a mixture of diastereomers in a 1:6 ratio (crude $^1$H NMR). Product formation was confirmed by spectral analysis.

**Scheme 3-19: Synthesis of compound 38**
Protons for C6 were observed at 8.54 and 8.46 ppm as singlets for both isomers. Sugar protons were observed between 4.92 and 3.78 ppm. Signals for the phenyl groups were identified in the aromatic region between 7.73 to 7.37 ppm. Signals for the isopropylidene, methyl and tert-butyl groups were observed between 1.66 to 1.01 ppm. This data was further supported by $^{13}$C NMR and HRMS analysis (Figure A-43 and A-44).

After the successful synthesis of 38, the C1' hydroxyl group was reduced to lactol 39 using L-selectride in the presence of ZnCl$_2$ to give 39 as a single isomer (Scheme 3-20). This reaction gave lower yields than reported for the related substrates. This may be partly due to the lower $\alpha/\beta$ ratio of 38, compared to the reported $\alpha/\beta$ ratio (1:8). To confirm this, we synthesized the lactol using the same protecting group strategy reported by Hanessian’s group shown in Scheme 3-21. Even in this case, lower yields were observed (65%) compared to published yields (85%). The issue which led to the lower yield could not be identified. From the literature it could be concluded that this reaction is not facile under all conditions. Other investigators indicated that this procedure failed to produce the desired lactol under the same conditions.$^{64}$ Hence, RedAl was used as a substitute for L-selectride/ZnCl$_2$. This reaction is however not stereospecific, hence the formation of two isomers was observed.$^{64}$
This reaction was successfully reproduced, however the yields were low. A definite advantage of this synthetic approach is the fact that a single isomer is obtained with a comparable yield using different protecting groups. The formation of 39 was confirmed by

Scheme 3-20: Synthesis of protected pseudouridine 41 using a new protection strategy
NMR and HRMS (Figure A-45, A-46 and A-47). A doublet at 5.25 ppm for H-1' clearly indicates the formation of 39. A signal for H-6 was observed at 8.30 ppm as a singlet. Along with this, signals for sugar protons were observed between 4.36 to 3.08 ppm. In addition, signals for all protecting groups were observed in the region between 1.61 to 1.10 ppm. The chemical shifts were compared to literature values to confirm the identification of each isomer.

Cyclization of 39 under Mitsunobu conditions using DIAD and Ph₃P gave 40 in ~ 60% yield. These reactions never went to completion resulting in lower yields. The addition of excess reagents made purification of the product difficult. Increasing the reaction time to room temperature slightly improved the yield. Formation of 40 was supported by the observation of a shift from 5.25 ppm for the H-1' to 4.98 ppm in 39 (Figure A-48 and A-49). Signals for all protons of the nucleoside were identified and additional support was obtained from HRMS (Figure A-50). After successful synthesis of 40, the 5'- t-BuPh₂Si protecting group was successfully cleaved by treatment with TBAF at 4 ºC for 1 hour. Purification by silica gel column chromatography gave pure 41. The disappearance of the NMR signals for the phenyl group between 7.74 - 7.37 ppm, and the tert-butyl group at

Scheme 3-21: Coupling and reduction reactions using bis-acetal
1.08 ppm supported the formation of the product (Figure A-51 and A-52). High resolution mass spectrometry confirmed product formation (Figure A-53). After successfully obtaining protected β-pseudouridine 41, we applied the strategy used for the synthesis of C5'-pivaloyluridine (27) to the synthesis of 43.

Scheme 3-22: Scheme for the synthesis of C5'-aldehyde and cyanohydrin

Figure 3-16: Pseudouridine C5'-hydrate
Oxidation of the 5'-hydroxyl to aldehyde (42) was carried out using the Dess-Martin Reagent (Scheme 3-22). Similar to the uridine-5'-aldehyde (49), aqueous work up was avoided for this reaction. However, a crude NMR of the product showed the formation of two nucleosides. One was the desired aldehyde 42 and the other the analogous undesired hydrate shown in Figure 3-16. Purification to remove the unwanted hydrate by silica gel column chromatography resulted in poor yields (50%). Efforts to optimize the yield have been unsuccessful so far. Formation of 42 was supported by NMR and HRMS (Figure A-54, A-55 and A-56). A strong signal for the aldehyde proton at 9.72 ppm confirmed the formation of the product.

Disappearance of the H-5' and H-5" signals in the region between 3.89 and 3.76 ppm further supported product formation. A signal for the carbonyl group of the aldehyde at 200 ppm in $^{13}$C NMR strengthens the assignment for the formation of the desired aldehyde. When HRMS was carried out by dissolving the compound in methanol, a peak was observed at
m/z 449.2246 which is the sodium adduct of expected methyl hemiacetal derivative of \(42\) (Scheme 3-23).

Treatment of \(42\) with a catalytic amount of 18-Crown-6 and KCN followed by the addition of 2.5 equivalents of tert-butyldimethylsilyl cyanide resulted in the formation of the TBDMS protected cyanohydrin \((43)\) as a mixture of isomers in a 4:5 ratio. This ratio is the same as the ratio observed in the cyanohydrin product of uridine \((34)\). The presence of the tert-butyl protecting groups on the base did not affect the diastereomeric ratio of the product. This reaction gave only 41% yield. Efforts are currently underway to optimize this reaction. Though, the yields are low for the above two reaction steps, successful synthesis of \(42\) and \(43\) confirms that our approach for the synthesis of C5’ pseudouridine is feasible.

Formation of \(42\) was confirmed by NMR (Figure A-57 and A-58) and HRMS. Disappearance of the signal at 9.72 ppm for the representative aldehyde confirmed the consumption of the starting material. Two singlets were observed at 8.29 and 8.21 ppm for H-6 of two diastereomers of the product. Two doublets were observed at 5.01 and 4.97 ppm corresponding to the H-1’ of both isomers. Additionally, protecting groups for the base and ribose sugar were observed between 1.64 ppm to 0.18 ppm. Carbons for the cyano group which is characteristic for this product were observed in the \(^{13}\)C NMR at 118.16 and 117.80 ppm. This evidence sufficiently supports product formation. The product identity was further supported by HRMS (Figure A-59).

Compound \(43\) was subjected to alkylation with \(t\)-BuLi and the intermediate imine was hydrolyzed by the addition of 1N HCl until the pH reached between 2 and 3. It was assumed that at lower pH, the nucleoside would be deprotected, so an additional reaction step for
deprotection could be avoided. However, during the synthesis of 36 removal of the TBDMS and isopropylidene groups was not observed. In the case of pseudouridine, it was assumed that the base protecting groups would be removed. Surprisingly, we did not form the expected product either protected or deprotected, in fact the compound observed was 2', 3'- isopropylidene protected uridine. This compound was completely unexpected, however its formation was confirmed by $^1$H NMR and ESI-MS. The proton NMR showed the presence of two base protons, which are present in uridine. Formation of the $t$-butylketone on was not observed.

This may result from the loss of the tert-butyl groups on the base under acidic conditions leading to ribose ring opening under these conditions which subsequently lead to the formation of pseudouridine degradation products (Scheme 3-24). In this case, the ribose ring was found to be intact.\textsuperscript{65,66}

ESI-MS showed significant peaks at m/z 540.0 and 563.0. Though this information did not help to elucidate the structure of the degradation product, it is supported by Cohn’s work that shows that isomerized products do not lose any carbon atoms. More experimental

\textbf{Scheme 3-24}: Isomerization of pseudouridine in acid
evidence is needed to understand this type of degradation. Also new protocols must be developed for the synthesis of this molecule.
Chapter 4

Conclusions

This study focused on understanding oxidative damage to RNA through studies on uridine and its C-glycoside analog pseudouridine. Our study aimed to generate C5'-radicals on both nucleosides and understand the fate and mechanisms of radical derived products. We successfully established the synthetic strategy to obtain a C5'-uridinylradical precursor and subjected this radical precursor to Norrish type I photocleavage. Products obtained through the generation of the C5'-uridinylradical were analyzed by HPLC and mass spectrometric methods. The C5'-uridinylradical precursor was photolyzed under anaerobic conditions in the presence of two hydrogen atom donors tri-\(n\)-butyltin hydride and the biologically relevant H-donor glutathione. Formation of the radical reduction product, uridine (26) and the base elimination product, uracil (57) were observed in the presence of glutathione and the yields were found to vary depending on the concentration of glutathione. In the absence of an H-donor, the base elimination product, uracil was the major product. In addition to these products, uridine-5'-aldehyde and uridine-5'-hydrate were observed under aerobic condition in the absence of an H-donor. These results conclude that, unlike deoxyribonucleosides, generation of C5’-radicals in uridine leads to base elimination. From this we can say that, C5'- RNA radicals may lead to more damage than in DNA. However, further studies are needed before coming to any final conclusion.
Furthermore, pseudouridine was successfully synthesized by altering the protecting group strategy and previously used by other groups to obtain this substrate was found to be beneficial for the later steps of the synthesis. Successful introduction of a protected cyanohydrin at C5'-position was achieved. Some of these reactions need to be optimized for higher yields. Progress towards the synthesis of a C5'-pseudouridinylradical precursor is ongoing. Once this radical precursor is in hand, it will be subjected to the same photolytic conditions as the C5'-uridinylradical precursor. Comparison of the radical derived products from these two precursors may provide some key information on RNA oxidation and then possible protective role of pseudouridine towards this damage.
Chapter 5

Future Work

5.1 Synthesis of C5'-pivaloyluridine H-phosphonate

Through this work the C5'-uridinylradical precursor was successfully synthesized and the fate of the radical was studied under anaerobic and aerobic conditions. The next step for this work is to incorporate the radical precursor into RNA oligomers and study the fate of

![Scheme 5-1: Synthesis of C5'-pivaloyluridine H-phosphonate](image)

the radical under physiologically relevant conditions.
Because RNA nucleosides have an additional hydroxyl group at the C2'-position when compared to DNA, synthesis of a monomer suitable for incorporation into oligomers is more difficult than DNA. Especially, the installation of the protecting group at the C2'-hydroxyl group. Also, our radical precursor has a C5'-tert-butyketone group which can make the synthesis of a suitable monomer difficult. Keeping these issues in mind, a possible route for the synthesis is proposed (Scheme 5-1). In order to incorporate a modified radical precursor into an oligomer a phosphorus containing group has to be installed onto the C5'-pivaloyluridine. For this we chose to utilize an H-phosphonate group.

Synthesis of the modified precursor starts with the protection of C5'-uridinylradical precursor 27, with TBDMS groups. Ogilvie showed that the protection of substrates like 27 at C5' and C2' by TBDMS groups is more selective in pyridine.\textsuperscript{67} Hence, this approach will be applied for the protection strategy. Later deprotection of the C5'-TBDMS group will be achieved by treatment with tetra-butylammonium fluoride at low temperature, so that loss of the C2'-TBDMS group can be prevented. Compound 112 will be obtained by the careful addition of DMTrCl in the presence of pyridine. Compound 112 will be converted to 114 using protocols developed by our group and others.\textsuperscript{42,68} Employing the H-phosphonate will be beneficial in this study over the commonly utilized phosphoramidite, because, the H-phosphonate is a small group eliminating the interference of bulky protecting groups of phosphoramidite in the synthesis. Synthesis of DNA/ RNA oligomers using phosphoramidite chemistry is a well-established technique. Phosphoramidites have a phosphoamide moiety, which is activated by the addition of a tetrazole activator. The activated phosphoramidite is recognized by the hydroxyl group of
the incoming nucleoside. **Scheme 5-2** shows a representative example for the synthesis of the pseudouridine phosphoramidite. Although, phosphoramidites are widely used for the synthesis of oligomers, it is not a choice of interest in this study mainly due to its size. Therefore, the H-phosphate group has been chosen to be installed at the C5'-position.

### 5.2 Synthesis of the C5'-pivaloylpseudouridine H-phosphonate

Similarly, the same strategy will be applied for incorporation of the C5'-pseudouridine radical precursor into oligomers. The Chow group approach to the synthesis of suitable precursors for incorporation into oligomers utilizes a novel protection-deprotection strategy.\(^{69,70}\) (**Scheme 5-2**). However, in their work the C5'-position was not blocked by any group, therefore the phosphoramidite approach worked well. As mentioned before, due to the presence of the C5'-tert-butylketone group, using phosphoramidite may not be a reasonable choice in our study. Therefore, in the case of the two radical precursors, the H-phosphonate derivatives were chosen. In addition, Chow et al. did not protect the amino groups of the nucleobase. This indicates that these groups are stable under the conditions utilized for the synthesis of the oligomer.
In this study, oligomer synthesis will be carried out using reverse automated DNA/ RNA synthesis. Normally DNA/ RNA synthesis occurs in the 5' to 3' direction, and in this study, the 5' position has been sterically blocked by a tert-butylketone which can interfere with the normal synthesis of the oligomer. Therefore, reverse automated DNA/ RNA synthesis (3' to 5') will be employed.

**Scheme 5-2:** Synthesis of Pseudouridine phosphoramidite by Chow et al. 69
5.3. Isomerization of C5'-uridylnylradical precursor (27) to C5'-pseudouridylnylradical precursor (97) using pseudouridine synthases

The strategy developed for the generation of the C5'-radical in both uridine and pseudouridine is the same. Therefore, it would be beneficial to simply isomerize the C5'-uridylnylradical precursor to the C5'-pseudouridylnyl radical precursor by enzyme catalysis using pseudouridine synthase (Scheme 5-1). As the ultimate goal of this project is to study the fate of C5'-radicals of uridine and pseudouridine in oligomers, it is also important to find ways to reduce the number of synthetic steps to get to the final stage. As the synthesis of the C5'-uridylnylradical precursor has already been established, we could use this as a starting material to obtain the C5'-pseudouridylnylradical precursor with the help of pseudouridine synthases. If this works well, at least ten synthetic steps will be eliminated.

![Scheme 5-3: Synthesis of C5'-pseudouridylnylradical precursor](image)

Application of enzyme catalysis for organic synthesis has been demonstrated in the literature.\(^{71,72}\) Enzymes are better catalyst than organic catalysts. They are more efficient, hence reactions take place very quickly and with higher yields. Furthermore, enzymes work
in buffer at neutral pH, which is a more “green” reaction. Therefore, this is an excellent alternative for the efficient synthesis of the C5'-pseudouridinylradical precursor. There are five classes of pseudouridine synthases known. Studies showed that all the enzymes share the same protein fold, however different substrate specificities.\textsuperscript{73,74} Separation of the enzyme after completion of the reaction will be a very important consideration. Though at this stage, we do not have a complete understanding on the activity of the enzyme, this strategy may be very beneficial over traditional organic synthesis, and definitely worth looking into it.
References


Appendix A

Spectral data
Figure A-1: $^1$H NMR for compound 34
Figure A-2: $^{13}$C NMR for compound 34
Figure A-3: HRMS for compound 34
Figure A-4: $^1$H NMR for compound 36
Figure A-5: $^{13}$C NMR for compound 36
Figure A-6: HRMS for compound 36
Figure A-7: $^1$H NMR for compound 121
Figure A-8: $^{13}$C NMR for compound 121
Figure A-9: HRMS for compound 121
Figure A-10: $^1$H NMR for compound 27
Figure A-11: $^{13}$C NMR for compound 27
Figure A-12: HRMS for compound 27
Figure A-13: MALDI-TOF Spectrum for compound 27

Figure A-14: RP-HPLC for compound 27
Figure A-15: $^1$H NMR for compound 37
Figure A-16: $^{13}$C NMR for compound 37
Figure A-17: HRMS for compound 37
Figure A-18: MALDI-TOF Spectrum for 57 obtained from the photolysis of 27

Calculated mass (m/z) = 113.034 (M+H), 135.017 (M+Na), 150.990 (M+K)
Observed (m/z) = 112.910 (M+H), 135.115 (M+Na), 151.116 (M+K)
**Figure A-19:** HPLC for the photolysis of 36 in presence of $n$Bu$_3$SnH. Pink: Compound 36, Blue: Reduction product 37, Black: Photolysate of 36
Figure A-20: MALDI-TOF Spectrum for 37 obtained from the photolysis of 36.
Figure A-21: Representative chromatogram for the photolysis of 27 with 2 mM GSH at pH 7.0

Figure A-22: Representative chromatogram for the photolysis of 27 with 4 mM GSH at pH 7.0
**Figure A-23**: Representative chromatogram for the photolysis of 27 with 8 mM GSH at pH 7.0

**Figure A-24**: Representative chromatogram for the photolysis of 27 with 16 mM GSH at pH 7.0
**Figure A-25**: Representative chromatogram for the photolysis of 27 with 24 mM GSH at pH 7.0

**Figure A-26**: Representative chromatogram for the photolysis of 27 with 32 mM GSH at pH 7.0
Figure A-27: Representative chromatogram for spiking of the standards Uridine and Uracil with photolyysate of 27 obtained with 32 mM GSH at pH 7.0
Figure A-28: MALDI-TOF Spectrum for the photolysate of 27 with 24 mM GSH at pH 7.0

Calculated mass (m/z) = 113.034(M+H), 135.017(M+Na), 150.990(M+K)
Observed (m/z) = 112.954(M+H), 135.119(M+Na), 150.157(M+K)
Figure A-29: MALDI-TOF Spectrum for the HPLC fraction of photolysate of 27 with 24 mM GSH at pH 7.0
**Figure A-30:** Representative chromatogram for the photolysis of 27 with 0 mM GSH at pH 3.5

**Figure A-31:** Representative chromatogram for the photolysis of 27 with 2 mM GSH at pH 3.5
Figure A-32: Representative chromatogram for the photolysis of 27 with 4 mM GSH at pH 3.5

Figure A-33: Representative chromatogram for the photolysis of 27 with 8 mM GSH at pH 3.5
Figure A-34: Representative chromatogram for the photolysis of 27 with 16 mM GSH at pH 3.5

Figure A-35: Representative chromatogram for the photolysis of 27 with 24 mM GSH at pH 3.5
Figure A-36: Representative chromatogram for the photolysis of 27 with 32 mM GSH at pH 3.5
Figure A-37: MALDI-TOF Spectrum for the HPLC fraction of photolysate of 27 in water

Calculated (m/z) = 245.076 (M+H), 267.059 (M+Na)
Observed (m/z) = 245.095 (M+H), 266.998 (M+Na+)
Figure A-38: ESI-MS Spectrum for the HPLC fraction of photolysate of 27 with 8 mM GSH in water
**Figure A-39**: MALDI-TOF Spectrum for the crude photolysate of 27 under aerobic conditions without a hydrogen atom donor.
Figure A-40: MALDI-TOF Spectrum for the crude photolyasate of 27 under aerobic conditions without a hydrogen atom donor

Calculated (m/z) = 113.034 (M+H+)
Observed (m/z) = 113.057 (M+H+)
Figure A-41: Representative chromatogram for the photolysis of 27 with 16 mM GSH in water
**Figure A-42:** $^1$H NMR for compound 38
Figure A-43: $^{13}$C NMR for compound 38
Figure A-44: HRMS for compound 38
Figure A-45: $^1$H NMR for compound 39
Figure A-46: $^{13}$C NMR for compound 39
Figure A-47: HRMS for compound 39
Figure A-48: $^1$H NMR for compound 40
Figure A-49. $^{13}$C NMR for compound 40
Figure A-50: HRMS for compound 40
Figure A-51: $^1$H NMR for compound 41
Figure A-52: $^{13}$C NMR for compound 41
Figure A-53: ESI-MS for compound 41
Figure A-54: $^1$H NMR for compound 42
Figure A-55: $^{13}$C NMR for compound 42
Figure A-56: HRMS for compound 42
Figure A-57: $^1$H NMR for compound 43
Figure A-58: $^{13}$C NMR for compound 43
Figure A-59: HRMS for compound 43