Probing Structure for Photophysical Properties Through Synthesis of Novel Nucleosides

Britney Rodgers

University of Denver

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PROBING STRUCTURE FOR PHOTOPHYSICAL PROPERTIES THROUGH
SYNTHESIS OF NOVEL NUCLEOSIDES

A Dissertation
Presented to
the Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Brittney Jean Rodgers
June 2014
Advisor: Dr. Byron W. Purse
Abstract

A brief history of nucleosides and their photophysics provides the background information for the discussion of synthesis, photophysics and incorporation of fluorescent nucleotide analogs during enzymatic DNA synthesis. Through the use of nucleoside starting material, it is possible to synthesize a number of modified nucleotides for use as fluorescent probes in biochemical research. Synthetic endeavors into the synthesis of these and related molecules from nucleobase starting materials are also presented. The synthesis of these molecules involved re-development of the synthetic routes to broaden substrate scope by overcoming the changed reactivity resulting from the presence of additional functionality.

While many highly useful fluorescent probes exist today, there remains to be filled significant gaps in performance. Brighter probes, probes that emit at various wavelengths while maintaining fluorescence when present in folded biomolecules (proteins, nucleic acids), and probes that work together as a nucleic acid FRET pairs are still needed.

In the work described in this thesis, a series of cytidine analogs that are closely related have been synthesized and investigated to elucidate trends in effects on photophysical properties by the electronic character and position of substituents. The location of substitution as well as the surrounding environment has an influence on how the introduction of new functionality affects the photophysical properties of the
chromophore. Tricyclic cytosine skeletons have been substituted to produce analogs that are brighter, and more or less sensitive to their environment.

These substitutions affect not only the photophysical properties of the compound but also the tautomeric equilibrium between cytosine and tyrosine-like hydrogen bonding patterns. This equilibrium between hydrogen positions controls the base pairing properties of the compound and how it is treated by cellular machinery during incorporation in nascent nucleic acids. Incorporation studies reveal that the substitution affects the incorporation efficiency for templates containing different hydrogen bonding partners.
Acknowledgements

I would like to acknowledge and thank my advisor Dr. Byron Purse for his guidance throughout this work. Thanks to my committee: Dr. Kutateladze, Dr. Eaton, Dr. Margittai, and Dr. Barbee. Members of the faculty of the University of Denver have had a great impact on my scientific understanding, and for this I am very grateful. I would also like to thank collaborators Dr. Robert Kuchta, and Dr. Gudrun Stengel for the biochemistry support on the project and instruction in photophysical experimentation. Thank you to Dr. Miroslav Kvasnitsa for all of the practical organic chemistry knowledge that I have gained, and all the other members of the Purse group past and present for all the help they provided. My undergraduate research advisor Dr. Rongson Pongdee is the reason that I came to graduate school, thank you for the inspiration. I thank Darrell Hundley, Christine Stutzman, Laurel Shurtleff, and Irene Occhiello for helping with anything and everything. Without the equipment of the Eaton research group and the Grotjan research group, none of this would have been possible. My fellow graduate students for the incredible support and empathy, I thank you. All of the undergraduate students that I have been lucky enough to work with have made this project into what it is, thank you to them. Thank you to the NIH for grant 1R15 GM093943-01 which provided the funding for this research.

Most importantly the encouragement, love, and support that has carried me through comes from my family: William Rodgers, Dana and Doug Mitchell, Robert III and Krystal Mitchell, Kimber and Blaine Cunningham, Jodi Rodgers, Ann Mitchell, and many more whom I am deeply appreciative of. Thank you to whom made all this possible.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>iPr</td>
<td>1,3-bis(2,6-diisopropylphenyl)imidazol-2-ylidene</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>2AP</td>
<td>2-aminopurine</td>
</tr>
<tr>
<td>MesCl</td>
<td>2-Mesitylenesulfonyl chloride</td>
</tr>
<tr>
<td>DMTTrCl</td>
<td>4,4-Dimethoxytritylchloride</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>AMM</td>
<td>Accurate mass measurement</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>Ac$_2$O</td>
<td>Acetic anhydride</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AlCl$_3$</td>
<td>Aluminum chloride</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>Ammonia</td>
</tr>
<tr>
<td>BOP</td>
<td>Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>PYBOP</td>
<td>(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>tC$_7$</td>
<td>Carbon tricyclonucleoside</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DBH</td>
<td>Dibromodimethylhydantoin</td>
</tr>
<tr>
<td>DME</td>
<td>Dimethoxyethane</td>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<td>Dimethylsulfoxide</td>
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<tr>
<td>Boc$_2$O</td>
<td>Di-tert-butyl-dicarbonate</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
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<td>Ethyl acetate</td>
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<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
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<tr>
<td>FBA</td>
<td>Fluorescent nucleobase analogs</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<td>G</td>
<td>Guanosine</td>
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<tr>
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<td>Hertz</td>
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<td>HMDS</td>
<td>Hexamethyldisilazane</td>
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<td>HMPA</td>
<td>Hexamethylphosphoramidate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
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LUMO
mRNA
MeOH
μL
μM
mg
mL
mmol
M
NCS
NMN
NMP
nm
NAD
BSA
NMR
tC°
Pd-C
PBS
PCl₅
PMT
QY
RD
RNA
SNP
tBuOOH
TBSOTf
THF
TLC
T
tRNA
tC
TEA
TEAA
TEAB
Tf₂O
TIBS-Cl
TMP
TMS-Cl
TMSOTf
PPh₃
H₂O

Lowest unoccupied molecular orbital
Messenger ribonucleic acid
Methanol
Microliter
Micromolar
Milligram
Milliliter
Millimole
Molar
N-Chlorosuccinimide
N-methyl imidazole
N-Methylpyrrolidone
nanometer
Nicotinamide adenine dinucleotide
N,O-Bis(trimethylsilyl)acetamide
Nuclear magnetic resonance
Oxygen tricyclic cytosine
Palladium (10%) on charcoal
Phosphate buffered saline
Phosphorus pentachloride
Photomultiplier tube
Quantum Yield
Reichardts dye
Ribonucleic acid
Single nucleotide polymorphism
tert-Butoxide
tert-Butylsilyl trifluoromethanesulfonate
Tetrahydrofuran
Thin layer chromatography
Thymidine
Transfer ribonucleic acid
Tricyclic cytosine
Triethylamine
Triethylammonium acetate
Triethylammonium bicarbonate
Trifluoromethanesulfonic anhydride
Triisobutylsilyl chloride
Trimethylphosphate
Trimethylsilyl chloride
Trimethylsilyl trifluoromethanesulfonate
Triphenylphosphine
Water
Chapter One: Introduction of Key Concepts

Nucleotides

Scientific interest in nucleotides dates back over 200 years which was long before the fundamental importance of these molecules was known. Karl Scheele discovered uric acid in 1776. This seminal discovery was followed many years later by identification of the other nucleobases. Guanine’s discovery in 1845 was followed more quickly by the precursor to adenine in 1847, thymine in 1893, and finally cytosine (which is important in the context of the molecules in this work) in 1894. The study of the acid resistant portion of surgical pus “nuclein” began in 1868 resulting in the term that would lead to the name used for these compounds today. The term nucleic acid (for nucleotides and their polymers) was suggested in 1889 by Altmann and Levene, and Mandel finally introduced the term nucleotide in 1905. First success in the synthesis of a phosphate containing nucleotide was achieved by Fischer in 1914. Synthesis without the phosphate groups can be easier, as will be shown in this work. The first published synthesis of cytosine without phosphate or ribose, the nucleobase, came from Wheeler and Johnson in 1903 (figure 1-0-1). This was not long after the correct structure proposal of the compound by Kossel and Stendel in 1902.
Nucleotides have a multitude of roles in the cell and are intricately tied to many processes. They are primarily the building blocks for the holders of genetic information but they also play a role in metabolism, signaling, and enzyme activity. Adenine and cytidine are weak bases, uridine and thymidine are acids and guanosine is amphoteric. Due to the variety of processes involving these molecules, probes that can mimic natural nucleobase structure are highly useful biophysical tools. Probe development is possible because these monomers and their corresponding macromolecules can be manipulated and modified through synthesis or with enzymatic activity. Nucleotides assemble into nucleic acids by rules that are understood and the geometries of these structures are predictable. The resulting polymers are capable of binding a wide range of compounds, and even catalyzing reactions. It has been recognized for some time that changes to the nucleotides will change the absorbance spectra of these compounds. Additionally, when making synthetic modifications to nucleotides it is important to keep in mind that the bond lengths and the bond angles will be different than the natural nucleotide, orientation with respect to the glycosidic bond will be affected, and the conformation of the phosphodiester bond can also change. These changes are important because slight

Figure 1-0-1: Original Cytosine Synthesis. Nucleobase structure was confirmed through its synthesis by Wheeler and Johnson in 1903.
variations in structure can have a significant effect on the functionality and recognition by cellular machinery. The earliest synthetic analogs were those that were modified versions of adenosine. Intercalating nucleotides have been synthesized, e.g. nucleobases that are stretched through the expansion of the $\pi$ system like those in this study. Other large $\pi$ systems are seen in ligandosides which are fluorescent bases that can be ligands for metal detection and are tolerated after incorporation into oligomers.\textsuperscript{10}

The nucleobases act like the functional groups of a molecular assembly which is the DNA helix. They align perpendicular to the backbone and are in direct $\pi$-$\pi$ van der Waals contact.\textsuperscript{11} Base pairing occurs when complimentary nucleobases on the same or different nucleotide strands bind through hydrogen bonds. Watson-Crick pairs form through size complementarity as well as a matching hydrogen bond pattern.\textsuperscript{12} The hydrogen bond pattern is influenced by the tautomeric state of the nucleotides. The bases of the nucleotides can exist in several tautomeric forms, the natural occurrence of which is dictated by the presence of the exocyclic hydroxyl and amino substituents. The diketo tautomer predominates in uridine (U) and thymidine (T) while the keto-amino is favored in cytidine (C) and guanosine (G), and the amino form is dominant in adenine (A).\textsuperscript{8}

Loops and bulges in oligonucleotides distort the stacking of the bases and can change the local environment of a nucleotide. Bulges are formed when one strand is longer than another in the duplex. Loops occur when there are consecutive mismatches between the strands or mismatches at the end and can be symmetric or asymmetric.\textsuperscript{13} RNA has a larger amount of structural diversity than DNA does, and individual unpaired bases are common in structured RNA. The high occurrence of loops, bulges, and
mismatches in RNA are essential for the function of RNA.\textsuperscript{14} Contrary to this
preponderance of unstacking in RNA, A form DNA is a nucleic acid with a higher
occurrence of stacking from a large amount of $\pi$ system overlap. B form DNA appears in
the middle with not as significant stacking as A form DNA but without the high
occurrence of mismatch seen in RNA. These stacking differences have an influence on
the electronic properties of the nucleotides.\textsuperscript{15}

The fraying of base pairs at the terminus of the duplex makes studying this region
more difficult than bases elsewhere in the helix.\textsuperscript{16} At the terminus, the base pair is open a
significant portion of time and this causes the neighboring base pair to experience a
terminus like environment. This behavior of the terminus causes the second base pair to
be more open and affect the next pair and so on and so forth.\textsuperscript{17} Robust synthetic
nucleosides will be required to study the differing environments in all areas of the helix.
Fluorescence of Nucleotides

Fluorescence preceded the nucleic acid history that was described in the previous section, and therefore the photophysical properties of nucleic acids and components have been under investigation almost since discovery. Using fluorescence spectroscopy one can obtain a large sample signal and a low background fluorescence so this technique is used to study a number of biosystems in which other experimental background signals could be significant. Fluorescent probes give access to structural elements of DNA-protein interactions and molecule recognition that would otherwise be unobtainable because probes substitute for the natural counterpart. Data obtained can give structural information of DNA on the molecular level. A fluorescent label can yield information about the structure, but because of the tether attachment to the molecule of interest it is always some distance away. Typically, organic chromophores reside in their excited states for less than twenty nanoseconds after the femtosecond long excitation processes. Collisional as well as other forms of quenching lead to a loss of emission intensity.

Non-fluorescent techniques are used in medicine daily, but are expensive and sometimes suffer from poor resolution. Additionally many of these techniques are unresponsive to physiological changes. The natural nucleobases have exceedingly low quantum yields (QY) and sub-picosecond fluorescent lifetimes, so modified nucleosides and other molecules have been synthesized to study these biomolecules with fluorescence. These short lifetimes are the result of rapid nonradiative decay. This decay has been attributed to the highly efficient internal conversion of absorbed energy to
harmless vibrational energy through nearly barrier-less conical intersections between vibrational states. Furthermore, participation of “dark states” has been observed. These non-emissive states feature out-of-plane polarized electronic transitions. Relaxations of these molecules are complex, and the decay is non-exponential. There is a strong wavelength dependence on the behavior of these compounds, and excess energy could potentially change the populations and the excited state dynamics. Guanosine, like the other natural bases is a poorly emissive compound. Additionally, it has also been observed that guanosine results in a decrease of the fluorescence from other chromophores in close proximity. This nucleobase has HOMO and LUMO orbitals of energies which make it possible to undergo photo-induced electron transfer. It has two strongly allowed $\pi\pi^*$ transitions that are in-plane polarized, but perpendicular to each other that are believed to be able to participate in electron transfer. Additionally, it has the highest number of heteroatoms and therefore most sites available for hydrogen bonding interactions. There is a “dark trap” in the energy landscape of guanine (figure 1-0-2). Generation of this state is usually rapid following excitation, but it can also be populated from the lowest energy state. It is likely that this state is the $\pi\sigma^*$, which is stabilized in protic solvents because of the stabilization of the nitrogen hydrogen bond.
Charge transfer from the amino in adenine is used to explain the observed red shifting of its emission. Similarly, another explanation for the quenching ability of guanine could be due to the intramolecular charge transfer from its amino to the chromophore. 8-oxo-guanine is produced upon oxidative stress, and it is shown to be an even better quencher than guanine. This not only provides a way to monitor oxidative stress, it supports the photoinduced electron transfer mechanism proposed. Interestingly, this damage response, if left unreversed leads to lesions, because the carbonyl oxygen leads to the presentation of the Hoogsteen face that mimics thymidine.

Fukuzumi has reported that the thermal electron transfer with one electron oxidants did not result in oxidation values that are significantly different between guanine and the other bases to explain why it acts so strongly as a reducing agent. The radical cation has been shown to be easily deprotonated, and this may be the explanation. The base portion in the nucleic acids are the chromophores, because the sugars and the phosphate backbone have their transitions at higher energy. Extensive π systems are
present in the natural nucleobases, and these are extended even further in the tricyclic systems investigated in this work. These $\pi$ systems lead to intense $\pi\pi^*$ transitions. From the number of filled and unfilled $\pi$ orbitals many $\pi\pi^*$ transitions are possible and these are the transitions likely observed in the wavelength range being investigated. The $n\pi^*$ transitions of the nonbonding oxygen and nitrogen electrons are typically buried by these other intense transitions.\textsuperscript{24} Higher ordered structures of nucleotides have a lower absorbance than the sum of the individual parts because the stacking interactions produce some hypochromicity.\textsuperscript{26} For oligonucleotides this typically results in a decrease of 20 to 40 percent, while a dinucleotide has a decrease of 0-13 percent.\textsuperscript{27} These electronic characteristics are useful in understanding fluorescent nucleobase analogs (FBA’s). Aromatic heterocycles such as nucleobases have been shown in previous work to be receptive to minimally perturbing substitutions designed to direct the photophysics.\textsuperscript{6} This receptiveness is exploited in the use of the compounds synthesized in this work to determine a substitution pattern which would give desired photophysical characteristics.

![Figure 1-0-3: Analog Y. Naturally fluorescent tRNA that was isolated in the 1970s and used to study tRNA using fluorescence.](image)

There are some naturally occurring modified bases that have fluorescence as a relaxation process. Analog Y was isolated and characterized in the 1970s (figure 1-0-3). Not only does this analog undergo fluorescence and phosphorescence relaxation, it undergoes photochemical decomposition.\textsuperscript{28} By 2007 there were 107 known naturally
occurring modifications of RNA nucleotides. A few of these have been shown to be emissive and their photophysics have aided investigations in tRNA, but most studies have necessitated use of external labels. 

Dyes that lie outside of the base stack can perturb the duplex and interfere with the interactions of nucleic acids which are of interest for investigation. This has been countered by the development of analogs that are substitutes for the natural bases while acting as a fluorescent probe of the nucleic acid. Pyrene has been attached to a nucleobase to take advantage of its significant fluorescence abilities. There are strong intramolecular interactions between the fluorophore and the base to which it is attached. The fluorescence lifetime is decreased for this fluorophore through its attachment as a label. Even with the negative impact on photophysics from the attachment of these labels to nucleotides, it is possible to have high absorptivities, high quantum yield and high photostability. Carboxy fluorescein for example can be conjugated to oligonucleotides. Fluorescence of this dye has been used to study the internalization of nucleic acids and the movement of these molecules within the cell. Extending the linkers of label molecules has been shown to provide intense tunable fluorophores.

It has been shown that endonucleases do not always cleave sequences which contain labeled nucleotides, perhaps from the bulk of the dye being in the way. These labels are also known to substantially increase the contour height and decrease the thermal stability of the duplex. In addition, the cumulative effect of multiple substitutions with these labels changes the solubility of the DNA in the organic phase and slows diffusion and hybridization action. There have also been some epistatic effects observed
with these types of substitutions. Attachment of the label to carbon 5 of the nucleobase through a short linker can place the dye in the minor groove. This placement was used in FRET (Forster resonance energy transfer) studies that were accurate out to 80 angstroms, to determine that Holliday junctions have different structures under different salt concentrations.\(^{36}\)

Using FRET it is possible to monitor the conformation changes and interactions of the system. For this type of energy transfer exploitation, it is important to consider the quantum yield of the donor chromophore, the spectral overlap between the fluorophores, the refractive index of the media, and the geometric factor. The geometric factor has to do with the similarity between the directions of the transition dipoles for the two molecules.\(^{37}\)

Time resolved Stokes shift spectroscopy was used to study abasic sites. There is a hypothesis that the increased flexibility present when there is an abasic site encourages the recognition of the site by cellular repair machinery. Experiments performed revealed that the DNA is a very polar environment.\(^{38}\) The environment of a chromophore is important to its performance. Solvation and hydrogen bonding cause a range of solvent effects on the electronic transitions of the nucleobases. The solvent environment of the molecule can change the structural arrangements that are accessible, it can change the energy order of the various bright and dark states, and it can promote other deactivating processes such as tautomerization and proton transfer.\(^{20}\)

It is possible to perform single molecule studies with fluorescence. Analogs that are able to be selective in their pairing partner are advantages for these studies.
Additional qualities of fluorescent base analogs that will be discussed in the following section make them more desirable than labels for these studies.\textsuperscript{39}
Fluorescent Base Analogs

Fluorescent base analogs make it possible to obtain conformational information on a picosecond timescale. These structural dynamics can be sequence dependent and FBAs allow for sequence specific experimentation. They are used in many applications because of their versatility and their close resemblance to the natural system. These analogs must have a minimal influence on the native structure of the DNA or RNA polymer to make them useful. A fixed and known orientation is also necessary, to understand the photophysical data that is to be collected from these probes. FBAs serve as real time probes of base stacking and base pairing and can be probes for diagnostic sequencing and molecular recognition.

A variety of probe options improves the experimental capabilities as well, because a compound can be selected for properties that are the most useful to the system under study. The stringent design criteria come from the helical stacking structure of the DNA. Size, shape, charge, polarity, and hydrogen bonding motif are all important for the determination of how useful a FBA will be and how much it will affect the natural structure of the duplex. Watson-Crick hydrogen bonds are not necessary for polymerase replication. The most important factor is that the base can fit into the active site of the enzyme. Polymerases impart restrictions based on steric that are much stricter than even the duplex.

FBAs have the ability to be firmly stacked, and report on the local structure with high sensitivity. Electrostatics are not as important when it comes to stacking, size and hydrophobicity are more important. Conversely, it is possible for analogs that are
present opposite an abasic site to be intrahelical or extrahelical, because they lack the hydrogen bonding partner to keep them within the stack. Sharp emission decay can be indicative of a flattening of the chromophore. The combination of emission decay and duplex stability can give information about the intrahelical or extrahelical location of the FBA. It is generally observed that FBAs that are found to be opposite these empty sites have an increase in quantum yield.\textsuperscript{43} Studying the non-nucleic acid functions of nucleotides is possible with fluorescent base analogs as well. There are NAD analogs and cAMP analogs which are used to study the interactions of these signal molecules in the cell.\textsuperscript{10, 44}

Modification to the pyrimidine at the 5 position has been shown to be a structurally benign modification, therefore this is the position where a lot of fluorescence enhancing modifications have been made to form FBAs.\textsuperscript{45} Substitution at this position on uridine and cytosine does not change the hydrogen bonding abilities and the substituents are pushed into the major groove in higher order structures. These analogs do show problems with polymerization likely due to a clash with the substituent and the active site of the enzyme. In addition analogs have been made to function as metal ligands, so that the metal can create the base pair.\textsuperscript{11}

Addition of a benzene ring between the sugar and the purines or in-between the pyrimidine rings of the natural bases has been shown to impart fluorescence (figure I-0-4). Only slight quenching is observed upon hybridization of these compounds, which is quantitatively less than 15%. Having an A in the complement strand was shown to be enhancing towards fluorescence for all but the extended A analog. Structural distortion
that leads to self-quenching has been implicated as the cause of this anomaly. The thermal stability of a duplex containing these analogs increases, but there is disruption in the natural structure. This distortion has been explained by possible mutual intercalation, formation of an alternative duplex form, or the lack of any structure at all.\textsuperscript{22}

![Image of DNA bases](image)


One of the most well-known fluorescent base analogs is 2-amino purine (2AP) which has the adenine exocyclic amine in the 2 position instead of in the 9 position (figure 1-0-5). 2AP has a quantum yield of 0.68 with excitation at 292 nm and emission at 305 nm that is significantly reduced upon incorporation.\textsuperscript{33, 46} This intensity reduction is presumably due to collisional quenching.\textsuperscript{15} It is less efficient in base pairing than adenosine and can act as a guanine analog as well making it less selective in its hydrogen bonding partner. Perturbations of the duplex which are discussed below are also observed upon incorporation of 2AP.\textsuperscript{33} This analog was used to determine the denaturation dynamics of structured RNA. Differential denaturation methods were shown to have a different influence on the lifetime components of the fluorescence of this FBA.\textsuperscript{14} This FBA was also used to determine that the formation of the open complex is not the rate
limiting part of translation. The perturbations induced by the presence of this FBA were investigated. There was shown to be an increase in the rate of exchange with water of the hydrogen bonding hydrogens in the base pairs of the FBA and neighboring bases. There was no premelting of the area around the FBA in denaturation so it has not been implicated in interfering with this mechanism. The decrease in the base pair lifetimes would influence the binding kinetics of cellular enzymes, interfering with the analysis of these types of mechanisms. This analog was observed to bind A dependent riboswitches with the same affinity as adenine, making it useful for the study of this cellular genetic control machinery.

Another adenosine analog is 6MAP; this analog has a fluorescence decrease of 90 percent upon incorporation and an additional decrease by a factor of 2 or 3 following hybridization (figure 1-0-5). This analog is very sensitive to its surrounding bases and can provide information about its environment. This FBA was used to study A tracts, which are known to have helix bending as a result of the long stretch of adenines. Metals were observed to bind preferentially to these tracts, and the degree of bending of the helix was greater at high concentrations of ions.
3MI is a guanine analog that forms ground state complexes when purines are present on either side in a single strand and has a similar environmental sensitivity to 6MAP (figure 1-0-5).\textsuperscript{50} Fluorescent analogs of guanine were used in conjunction with other dyes to determine the cleavage specificity influenced by cofactors on the cellular machinery.\textsuperscript{51} Isoxanthopterin is a G analog with a high quantum yield and sensitivity to its environment that slightly disturbs the duplex hybridization (figure 1-0-5).\textsuperscript{52} Pteridines like Isoxanthopterin are also highly environmentally sensitive, and this sensitivity comes from a combination of hydrogen bonding, stacking, and neighboring base influence. Although selective excitation is easy, hydrogen bonding to cytosine is hindered, resulting in a reduction in oligo stability.\textsuperscript{33}

Pyrrolo-dc is selective for its partner, G, but like many of these analogs it has a quantum yield that decreases from free nucleoside to single stranded and to double stranded nucleic acids (figure 1-0-5).\textsuperscript{33} In a duplex system it has been shown that there is potentially greater accessibility of the conical intersections between the states. This allows for an increase in the competitive decay processes which is an explanation for the common decrease in quantum yield.\textsuperscript{31} To reach these intersections a great deal of structural variation is required. Another question that has been asked is whether interbase proton transfer affects the rate of decay of a base pair.\textsuperscript{20} A potential use for this is the detection of mismatch by the increase in quantum yield that results from the decrease in the rigidity of the probe.\textsuperscript{31} Some groups have used nonpolar, weakly hydrogen bonding analogs to probe the stacking interactions for surface area, electrostatics, polarizability and hydrophobicity to understand the environment mismatched bases experience.\textsuperscript{53}
There are nonpolar nucleoside isosteres which are close mimics to the natural bases but that cannot form hydrogen bonds. Benzene was substituted for the natural base as a non-selective stacking nucleoside, but it turned out to be highly destabilizing.\textsuperscript{11} Combining a small set of fluorophores like these isosteres can give properties that are not the same as the individual molecules.\textsuperscript{11} Exciplexes including those in oligonucleotides are more sensitive to quenching, because there are multiple available quenching positions. Non-covalent binding interactions affect these phenomena.\textsuperscript{54} A combination of probes where each one is detectable through a different channel is an experimentally useful tool.\textsuperscript{55}

Taking fluorescent base analogs to the next level are the fluorescent analogs that mimic the electronic structure of the natural bases. There are stringent design criteria for an analog to be isomorphic to the natural bases, and the relationship between the structure and photophysics is difficult to establish.\textsuperscript{45} It is believed that creating a donor type substitution that can interplay with the acceptor portion of the base can lead to a red shift and an increase in environmental sensitivity.\textsuperscript{45} Isomorph analogs can be used in fluorescence sequencing and that makes them applicable to single nucleotide polymorphism (SNP) detection. Applications in this area make fluorescent base analog development relevant to human health and personalized medicine. Structure and function elucidation, microenvironment detection, ligand binding studies, and other miscellaneous applications are made available through the use of these compounds.\textsuperscript{6}

FBAs have uses outside of biophysical tools to study cellular nucleic acid processes. Attaching fluorescent sensors to a DNA backbone provides water solubility to
molecules that previously had none, which increases their utility in the determination of photon flux.\textsuperscript{56} A long range charge transport through the $\pi$ stack has interesting biosensor applications. Metallo base pairs have been used to expand the electronic and magnetic function ability of these compounds.\textsuperscript{57} Some modified nucleobases show anticancer and antiviral properties.\textsuperscript{11} Extension of the aromatic portion of the nucleobases was investigated as a possible stabilizer of triplex formation. Triplex formation could be a way for antisense mRNA to overcome the lack of target association because of sequestration in a duplex. The alpha anomers prevented exonuclease activity on the strands studied. Although there was a preferential action of these bases with duplexes, the triple helix was not stabilized.\textsuperscript{58} Triple helices are also seen in the use of size expanded bases (figure 1-0-4). These bases can be fluorescent from the extension of the $\pi$ system which increases the size, but are not generally used as FBAs. Although these bases are destabilizing following incorporation into a natural helix, an all expanded system shows more stability by 12-25 °C.\textsuperscript{59} Interestingly, these are not as destabilizing as having no nucleobase at all.\textsuperscript{60} Stability is imparted by the fluorescent base analogs which represent the scaffold for the nucleosides presented in this investigation. These analogs are shown to be environmentally less sensitive than other quenchable analogs. Environmental insensitivity generally comes from a high QY as well as a single fluorescence lifetime. High control of orientation and position is also common in environmentally insensitive fluorophores.\textsuperscript{53}
Tricyclic Cytosine Analogs

Some analogs have a problem with base pairing and thus are more likely than the natural counterparts to be flipped out of the base stack. Conversely, tricyclic cytosine (tC) appears to be pulled closer to the stack of bases than cytidine (figure 1-0-6). The hydrogen bonds between tC and guanosine are as stable as those between cytidine and guanosine. The one drawback of this tight interaction is that binding events that naturally occur in the major groove may be potentially affected by the presence of the tricyclic FBA.\textsuperscript{39} Following incorporation of tC there is minimal change to the local DNA structure and the overall form remains B-DNA.\textsuperscript{39} Tricycle to tricycle stacking has been shown to be dramatically stabilizing.\textsuperscript{3} Thiol tC has a quantum yield that is insensitive to location of the FBA within single strand or double strand oligonucleotides. Variations in the sequence surrounding the FBA also do not affect the QY. The oxygen analog of this compound is similar to previously discussed FBAs which show some surrounding sequence dependence, sometimes significantly, on photophysical properties. Oxygen tricyclic cytosine (tC\textsuperscript{o}) shows a strong distance dependent quenching by guanine to the 5’ side. Both tC and tC\textsuperscript{o} show a single fluorescence lifetime even after incorporation which is different from other analogs whose number of lifetimes increases upon incorporation due to various degrees of interactions in the helix. This conformational rigidity combined with the parallel emission and absorbance dipoles make a combination of tricyclic cytosine analogs a likely FRET pair.\textsuperscript{42}

Oxygen tricyclic cytosine is predicted to be flexible but planar, while the switching of oxygen for sulfur creates a bend along the SN axis.\textsuperscript{61} The ability of the
additional benzene ring of tricyclic cytosine to invert about this axis is believed to be unaffected by the placement in the major groove.\textsuperscript{62}

In addition to these two parent compounds; tCnitro, which as the name would imply, has a nitro group at the 7 position of the nucleobase, all base pair with guanosine. The selectivity is not the same selectivity as the natural cytosine because there is some misincorporation opposite adenine in studies performed.\textsuperscript{63} Misincorporation has been attributed to the hydrogen bonding face displayed by the analog, as will be further discussed in Chapter 5 regarding biological behavior of nucleotide analogs.\textsuperscript{64} Misincorporation rates in RNA and DNA are different because of discrimination differences by the cellular enzymes.\textsuperscript{65}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{tricyclic_cytidine.png}
\caption{Tricyclic cytidines. The molecular skeleton on which substitutions were made to probe photophysical properties. X is a carbon, oxygen or sulfur atom. R is usually hydrogen, R1 can be hydrogen or hydroxyl. R2 and R3 are variable. Photophysics have been investigated on the parent compounds in many environments, as will be discussed in Chapter 4 regarding the characteristics of the novel nucleotides. In general, high quantum yields are maintained following incorporation and hybridization. Upon incorporation there is a slowing of the nonradiative decay processes, which allow for an increase in QY. Sulfur analog tC has a decay that is dominated by temperature dependent processes while the tC\textsuperscript{o} decay is composed of almost an equal mix of temperature dependent and independent processes that sum to be equal to the decay of}
tC.\textsuperscript{66} Beginning in single stranded structure, decay of tC\textsuperscript{o} becomes biexponential because of these multiple processes.\textsuperscript{33} Although fast decay is not ideal when it comes to the brightness of a fluorophore, it is believed to increase the photostability.\textsuperscript{66} The lowest energy absorption of tC is likely from a single transition polarized at an angle 35 degrees to the long axis of the chromophore.\textsuperscript{67}

Because tC\textsuperscript{o} shows an increase in cellular permeability, it inspired the synthesis of a tetracyclic analog that would potentially enhance hybridization.\textsuperscript{68} Another extension of the tC\textsuperscript{o} framework is the g-clamp analog. G-clamp forms an additional hydrogen bond with the oxygen 6 of the complementary G located on the Hoogsteen face.\textsuperscript{69} These molecules have inspired others and they inspired the work presented in this dissertation.
Goals

The work presented here builds upon the above knowledge of nucleosides and their fluorescence. Creating a small library of related molecules that would establish a potential pattern between substituent and photophysical properties was the goal of this work. This pattern could then be used to direct further synthesis of fluorescent nucleoside analogs for specific applications. Selecting a framework that provided the beneficial characteristics presented in the previous section, tricyclic cytidines were the compounds selected to be the basis of this family. Knowing that the tC and tC\textsuperscript{o} analogs maintain their fluorescence upon incorporation and hybridization,\textsuperscript{42} this investigation aimed to improve upon this chromophore by determining substituents that would cause an increased brightness and or a shift in emission to longer wavelengths. A great enough increase would make these compounds potential tools for single molecule studies. Changes in HOMO LUMO energy gaps that result in the absorbance of one analog overlapping with the emission of another would result in a potential FRET pair. These were important goals of the investigation. Substitutions that would cause the chromophore to signal a change in environment through a photophysical change would also be useful. Being such close mimics to the natural structure, these compounds would provide dynamic information for specific bases in a nucleic acid.

Developing a nucleobase directed synthesis for the tC\textsuperscript{o} compounds would be exciting for reasons that will be discussed in the chapter regarding that synthesis. Modifications to the established synthetic routes for these compounds to increase tolerance towards a variety of substitutions that inherently decrease reactivity are another
synthetic highlight of this work. Synthetic manipulations of the substituent to further manipulate the functionality of the chromophore were aims of both the library establishment and elaboration. As an alternative, nucleoside directed synthesis was also investigated to determine the best route to the production of novel florescent nucleosides.

With the knowledge that the tricyclic skeleton is compatible with cellular machinery, but with greater error rates, and the hypothesis that this may be a result of tautomeric equilibria, substitutions were made to investigate this property as well. Establishing a relationship between the substituent and the incorporation of the nucleotide opposite natural bases would help to prove this long studied mechanism. A substitution that disturbs the tautomeric ratio enough could allow for detection of the normally minor form.
Chapter Two: Nucleobase Directed Synthesis

Introduction

Derivatives of the tricyclic cytidines are potentially accessible by two approaches: synthesis of the modified nucleobase followed by (deoxy)ribosylation, or synthetic elaboration of a nucleoside precursor. Both approaches were investigated in this work because of their distinct merits. The former has the advantages of scalability because inexpensive starting materials, whereas the later avoids the need to make the sometimes challenging glycosidic bond that links the nucleobase to (deoxy)ribose. This chapter presents the nucleobase directed synthetic approach (the former) to three tricyclic cytosine analog groups (figure 2-0-1). Parent compound tC which has sulfur in the center ring, tC° which has oxygen in this position and tC© where the atom in question is a carbon are the three parent compounds. Not only are the unsubstituted analogs desirable, but substitutions to the framework that would influence fluorescence are installed synthetically on these molecules. Some of these substitutions are synthetic handles for the installation of additional functionality. Alkyl amines are common in fluorescent dye molecules, and were a desired functionality to install on these synthetic nucleosides.
A nucleobase directed synthesis is a reasonable method by which to create novel nucleoside analogs.\textsuperscript{2, 70, 71} It has benefits over a nucleoside directed synthesis because one does not have to worry about severing the glycosidic bond during the synthetic elaboration process. Additionally, a sugar does not have to be preselected prior to synthesis of the chromophore; it can be later attached to whatever moiety the experiment in question requires. Ribose, deoxyribose or some synthetically developed mimic thereof are all possible to append.

Having the nucleobase itself can make it easier to investigate the fluorescence properties as well. Because the sugar group has been shown to have little influence on the spectroscopic properties of these molecules, photophysical data can be acquired earlier in the synthesis to give an idea as to which analogs are the most promising.\textsuperscript{25} It is not all positives. As with many endeavors there are some drawbacks to a nucleobase directed synthesis as well. A protecting group strategy is necessary when dealing with the nucleobase starting materials because of the reactivity of the N1 nitrogen that would be attached to the sugar in a nucleoside directed synthesis. When the nucleobase is formed, and it is time to glycosylate, there is often a loss of material because glycosylation lacks anomeric selectivity (typically a problem for 2’-deoxyribose, but less so for ribose). Although some methods exist to isomerize these less desirable anomers to the ones

\textsuperscript{25}
desired, these methods typically lack broad applicability. Reactions developed for nucleosides are often not applicable to the synthesis of the nucleobases, as they may rely on the presence of the functionality in the ribose for other assistance besides the protection of the N1 nitrogen (this will be seen in the following chapter).

Through the method development for synthesis discussed in this chapter, many of these pluses and minuses of a nucleobase directed synthesis are illustrated. The synthesis of many intermediates on the route to these synthetic nucleosides is simplified through the use of a nucleobase directed approach. Unfortunately, the final synthetic steps for the completion of the tC\textsuperscript{o} chromophore become a major issue in the absence of ribose. Benzyl protecting group was robust under the conditions for formation of the modified nucleobase, but were difficult to remove when the time eventually comes for glycosylation. For the sulfur compounds (tC), a nucleobase directed approach is a method that is shown to be effective for derivative synthesis in this work. Further derivatives may be accessible my similar methods.
Tricyclic Cytosine Analogs

Tricyclic cytosine tC is synthesized as the nucleobase first and then glycosylated to form the nucleoside. The step by step synthesis of the unsubstituted compound is found in the literature, and it is by this method that the compound was synthesized for the fluorescence investigations done by the author. 2-Aminothiophenol is coupled to 5-bromouracil with the assistance of sodium carbonate in refluxing ethylene glycol (figure 2-0-2). The basic environment allows the deprotonation of the sulfur which acts as a nucleophile for substitution at the bromine on the C5 carbon of 5-bromouracil. Closure of the second ring through the formation of the secondary amine is accomplished at 75 degrees with hydrochloric acid in ethanol. Acid catalysis encourages the substitution of the nitrogen of the 2-aminothiophenol at the C4 carbonyl of the uracil in ring closure. The nucleobase is fluorescent so it is easy to distinguish the product from other reaction components during analysis and purification.

Figure 2-0-2: Synthesis of tricyclic cytosine nucleobase (tC). 2 Nucleobase synthesis resulted in improved yields from the previous nucleoside route.
The glycosylation of this chromophore was improved over the literature methods in the laboratory. The N1 of the nucleobase is activated for substitution through the use of bis(trimethylsilyl)acetamide and then the p-tolyl protected chlorine substituted 2-deoxyribose is added with tin tetrachloride (figure 2-0-3). The oxygen of the C14 carbonyl attacks the silyl group of the BSA and the lone pair of electrons from the N1 nitrogen are pulled into a double bond with the C14 carbon. Reformation of the carbon oxygen double bond allows the nitrogen to act as a nucleophile to attack the cation generated at the 1’ position on the deoxyribose from chlorine elimination. This chemistry traditionally used for the synthesis of ribonucleosides was useful for both deoxyribonucleosides and ribonucleosides with the ribonucleoside synthesis resulting in higher yields.\(^72\)

The ribonucleoside synthesis was accomplished through the use of a tetra acetyl ester ribose with trimethylsilyltriflate as the Lewis acid (figure 2-0-4). The presence of the acetyl oxygen on the 2 position of ribose can coordinate the intermediate cation and direct the substitution which leads to an increased yield of the desired anomer. Removal of the protecting groups, whether acetyl or tolyl for the ribonucleosides and deoxyribonucleosides respectively was accomplished using Zemplen deacetylation.\(^73\)

These conditions utilize sodium methoxide in methanol. The sodium methoxide acts as a
nucleophile to substitute at the protecting group for the sugar oxygens and quenching of the reaction with acetic acid replaces the protecting groups with hydrogens.

![Chemical Structures]

Figure 2-0-4: tC ribosylation. Glycosylation with and deprotection of the nucleobase synthesized as presented in 2-0-2 to form the ribonucleoside

A related synthetic method was used for the synthesis of the tC analogs which contain additional functionality at the 7 or the 8 position. The synthetic method development of these molecules is the material of the thesis of Nada El-Sharif and it will not be discussed here. The synthetic route to these substituted tricyclic cytosine analogs is presented here for reference; these methods were used to produce the molecules that were used in the fluorescence investigation in this work Chapter 4 (figure 2-0-5).

Substituted benzothiazole is opened to the corresponding 2-aminothiophenol through the use of refluxing hydrazine hydrate. The hydrazine nitrogen attacks the C2 carbon of the benzothiazole and the N3 nitrogen C2 carbon double bond takes the hydrogen from the positively charged hydrazine nitrogen. This process repeats itself to sever the resulting carbon nitrogen single bond generating the free amine at N3 and an imine between C2 and the hydrazine nitrogen. Attack at the C2 carbon by water breaks the imine and the double bond abstracts a proton from the incoming water molecule. Deprotonation of the resulting hydroxyl by the thiazole sulfur leads to the generation of acethydrazine and the substituted aminothiophenol. This aminothiophenol is unstable, and so it is oxidized with hydrogen peroxide to form the more stable disulfide intermediate. The sulfur of the 2-
aminothiopenol attacks the oxygen of the hydrogen peroxide releasing a hydroxide ion which can deprotonate the sulfur to form the sulfenic acid. This sulfenic acid sulfur is attacked by sulfur on another aminothiophenol to form the disulfide and releasing a hydroxide ion.

When ready to couple this 2-aminothiophenol to 5-bromouracil, it can be reduced back to the sulfide through the use of triethylphosphine with water in diglyme. The phosphorous of triethylphosphine attacks one of the sulfurs of the disulfide bond and releases the other as the sulfur anion. The oxygen of water attacks the phosphorous to begin the generation of triethylphosphine oxide. The sulfur anions remove the protons from the water oxygen. Coupling to 5-bromouracil and ring closure happen in the same fashion as that described for the parent compound above to afford the substituted tC nucleobases. These nucleobases are glycosylated to form the deoxyribonucleoside and deprotected under the same conditions as the unsubstituted compound.

![Figure 2-0-5: Synthesis of substituted tC deoxyribonucleosides. Changes to synthetic method from that of the unsubstituted compound were necessary because of starting material availability and stability.](image)

Using the newly installed substituent on the nucleobase as a synthetic handle for the installation of new functionality is a possible route to new and interesting compounds. Given the absence of a sensitive glycosidic bond, first attempts at modification of this position were made prior to the attachment of the ribose. The methoxy substituted tC
nucleobase which had the ether substituent in the 8 position of the nucleobase was easier to synthesize and the starting material was less expensive, so the 8-OMe-tC was the nucleobase upon which synthetic elaboration was attempted (figure 2-0-6). Reaction with boron tribromide in dichloromethane afforded the hydroxyl substituted nucleobase through the cleavage of the ether. Oxygen of the methoxy ether attacks the boron of the boron tribromide and one of the bromines from the intermediate attacks the carbon of the ether resulting in cleavage of the ether bond. Aqueous workup leads to water displacing the boron from what was the ether oxygen and protonating this oxygen as well as the bromine anions that result from the boric acid formation. It was possible to glycosylate this hydroxyl substituted tricyclic cytosine nucleobase through the usual method. Yields for glycosylation of the OH-tC were lower than those for the methoxy analog.

![Chemical structure](image)

**Figure 2-0-6: Synthesis of triflate tC nucleobase. Conversion of the methoxy nucleobase to an intermediate more reactive to substitution**

Activation of this hydroxyl group to substitution by new functionality is possible through the installation of a triflic group on the oxygen. Trifluoromethanesulfonic anhydride was reacted with the hydroxyl tC nucleobase in dichloromethane with triethylamine and this resulted in the complete conversion of the starting material. The oxygen of the alcohol is deprotonated by triethylamine and the anion attacks the sulfonic sulfur resulting in cleavage of the anhydride following sulfur oxygen double bond reformation. The NMR signals of the nucleobase hydrogens were difficult to identify.
even after purification, and the fluorine signals observed for the crude mixture were absent from the compound that was recovered from purification. To check the utility of these triflating conditions, the same conditions were used to install the triflate group on phenol on small scale in deuterated solvent and investigated by NMR. There was evidence of a reaction by fluorine NMR. Repetition of the reaction of hydroxyl tC nucleobase under these conditions was made with multiple additions of triethylamine and triflic anhydride. This reaction resulted in recovery of the product, as was confirmed by mass spectroscopy. The material was not recovered in an amount that facilitated the

![Figure 2-0-7: Buchwald Hartwig amination. Testing conditions for the conversion of chlorine substituted nucleobases to alkyl amine substituted nucleobases.](image)

glycosylation or the substitution of this triflic tC nucleobase.

An alternative approach to the installation of new functionality, especially amine functionality, would be using Buchwald-Hartwig amination and a chlorine substituted nucleobase (figure 2-0-7).

Chlorine tC\textsuperscript{0} nucleobases were targets of the synthesis of oxygen tricyclic cytosine as will be discussed in the following section. To test potentially suitable reaction conditions, allyl[1,3-bis(2,6-diisopropylphenyl)-imidazol-2-ylidene]palladium (II) chloride was combined with potassium tert-butoxide, diethylamine and chlorobenzene in dimethoxyethane.\textsuperscript{80} The oxyanion on the tert-butoxide attacks the double bond of the aryl palladium ligand and the electrons from the metal ligand bond are left on the metal while the chlorine leaves as an anion resulting in the palladium (0) for
catalysis, potassium chloride and aryl ether. Alternatively, the catalyst could be activated resulting in the same products through the oxidative addition of the oxyanion of the tert-butoxide following the loss of the chlorine anion. The oxygen of the tert-butoxide then attacks the aryl ligand and the metal ligand bond electrons are left on the palladium as the ether is eliminated. Oxidative addition of the chlorobenzene to the palladium catalyst begins the cycle. The tert-butoxide substitutes at palladium for the chlorine forming potassium chloride. The amine substitutes for the tert-butoxide and the oxygen anion can deprotonate the amine to bring it to neutrality. Reductive elimination results in the installment of the diethylamine functionality at the position on the benzene that was previously held by the chlorine. The volatile nature of the chlorobenzene starting material made this a difficult test reaction to monitor. 1-Chloronaphthalene, being less volatile, was easier to react under the above conditions and this starting material resulted in the expected diethylamine product. The yield was not high enough from this reaction to appear a promising course of action for the substitution of chloro-nucleobases to diethylamine nucleobases.

Installation of the diethylamino functionality directly into the nucleobase starting material was another route that was attempted, acknowledging that substitutions greatly complicate the synthesis of these nucleobases. Benzothiazole is commercially available with an amine substituent in the 5 or the 6 position. Following the synthetic route for

Figure 2-0-8: Synthesis of diethylamine benzothiazole starting material. Two methods to conversion of amine starting material to diethyl amine substituted nucleobase precursor.
substituted tricyclic cytosines found above (figure 2-0-5) these positions correspond to
the 7 or the 8 position of the resulting nucleobase product. The amine was converted to
the diethyl amine through alkylation with bromoethane and potassium carbonate in
dimethyl sulfoxide (figure 2-0-8). The substitution of the bromine on the ethyl group by
the amine on the benzothiazole is assisted by the basic environment. Carbonate can
deprotonate the aminal nitrogen after it substitutes for the bromine on the alkane.
Repetition of substitution leads to the diethyl product. This reaction afforded the desired
product but in a mixture of both the di and the monoethyl amines which was difficult to
purify. Alternatively, the amine synthesis was approached through a reductive amination
approach (figure 2-0-8). Acetaldehyde, sodium cyanoborohydride, and acetic acid were
combined with the starting material in acetonitrile. The acetic acid protonates the
carbonyl oxygen of acetaldehyde which increases the electrophilicity of this compound.
Nucleophilic attack by the aminal nitrogen of the benzothiazole occurs on the carbonyl
carbon of the protonated acetaldehyde. Proton transfer from the nitrogen of the amine to
the oxygen of the aldehyde creates a good leaving group which the nitrogen lone pair
pushes off. Formation of the double bond between nitrogen and carbon leaves the
nitrogen positively charged. Deprotonation of this amine provides the imine intermediate.
Reduced of this imine intermediate by the cyanoborohydride is the final step, and this
entire process repeats itself to form the diethylamine. The desired product can be isolated
from this reaction in modest yield even upon scale up. 6-Diethylamino-benzothiazole
(compound A figure 2-0-8) was subjected to the cleavage conditions used in the
substituted tricyclic cytosine synthesis discussed above (figure 2-0-5). Refluxing with
hydrazine gives the desired 5-diethylamino-2-aminothiophenol as evidenced by thin layer chromatography. Upon oxidation of the 5-diethylamine-2-aminothiophenol the desired disulfide product was not obtained. The possibility for over oxidation led to the attempted by pass of the oxidation and reduction of the aminothiophenol. In situ coupling to 5-bromouracil of the cleavage reaction mixture was attempted. The results of this reaction were inconclusive.
Tricyclic Cytosine Analogs Experimental:

3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one tC. 5-bromouracil (3.15 g, 16.6 mmol) was combined with sodium carbonate (1.76 g, 16.6 mmol) and 2-aminothiophenol (2.4 mL, 22.3 mmol) under N₂ and dissolved in ethylene glycol (10 mL). Under reflux the reaction was stirred for 1 hour. Dilution with water, followed by neutralization with acetic acid and filtration of the product with water, ethanol and diethyl ether washes resulted in the intermediate. Sodium hydroxide (0.67 g, 16.7 mmol) in water (33mL) was heated to 50 °C and the solid intermediate was added in portions. Neutralization with acetic acid followed by filtration with water, ethanol and diethyl ether washes resulted in intermediate. Hydrochloric acid (2.4mL 37%) was dissolved in ethanol (24 mL) and the above intermediate was added and refluxed for 24 hours. After filtration, the solid product was added to a solution of sodium hydroxide (50 mL 5%) and stirred for 5 minutes at 50 °C, filtered and dried to yield (418 mg, 1.9 mmol) product 12% yield. ¹HNMR ((CD₃)₂SO) δ 10.97 (s, 1H), 10.22 (s, 1H), 7.41 (s, 1H), 7.08-7.01 (m, 2H), 6.93-6.87 (m, 2H).
**tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside.** 3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (100 mg, 0.46 mmol) was suspended in dry acetonitrile (2.5 mL) in a Schlenk tube under nitrogen. Bis(trimethylsilyl)acetamide (130 μL, 0.53 mmol) was added and the reaction mixture allowed to stir at 50 °C for 1 h. The reaction mixture was allowed to cool to room temperature and 3′,5′-di-O-(p-toluoyl)-2′-deoxy-α-D-ribofuranosyl chloride (198 mg, 0.51 mmol) was added with stirring. The reaction mixture was cooled to 0 °C and tin(IV) chloride (11 μL, 0.092 mmol) was added dropwise. The reaction mixture was stirred at 0 °C, then allowed to warm to room temperature, and the progress was tracked by thin layer chromatography. The reaction was complete in 90 min. At this time, the mixture was diluted with ethyl acetate (15 mL), washed with a saturated sodium bicarbonate solution, and dried. Rotary evaporation followed by purification using flash chromatography (EtOAc in cyclohexane) yielded the pure β anomer of the product as a yellow solid. (114 mg, 0.221 mmol) in 48 % yield.

**1H NMR (CDCl3) δ:** 7.97-7.87 (m, 4H), 7.43 (s, 1H), 7.32-7.26 (m, 2H), 7.23-7.17 (m, 2H), 7.07 (ddd, J=8.0, 7.0, 1.9 Hz, 1H), 6.98-6.89 (m, 2H), 6.80 (dd, J=7.9, 1.2 Hz, 1H), 6.37 (dd, J=8.2, 5.5 Hz, 1H), 5.59 (dt, J=6.6, 1.7 Hz, 1H), 4.78 (dd, J=12.3, 3.0 Hz, 1H), 4.67 (dd, J=12.3, 3.2 Hz, 1H), 4.59 (q, J=2.8 Hz, 1H), 2.95 (ddd, J=14.5, 5.6, 1.6 Hz, 1H), 2.43 (s, 3H), 2.36 (s, 3H), 2.21 (ddd, J=14.6, 8.3, 6.4 Hz, 1H).
**tC 2′-deoxy-β-D-ribonucleoside.** tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside (100 mg, 0.175 mmol) was dissolved in methanol (5 mL) and a solution of 30 % NaOMe in methanol (96 μL, 0.517 mmol) was added dropwise. The reaction progress was followed by thin layer chromatography and found to be complete in 30 min. The reaction was quenched by the addition of acetic acid (50 μL, 0.872 mmol) and the solvent was removed by rotary evaporation. Purification by flash chromatography (CH$_3$OH in CH$_2$Cl$_2$) yielded the product as a yellow solid (53.0 mg, 0.157 mmol) in 90 % yield.\textsuperscript{1}HNMR ((CD$_3$)$_2$SO) δ 10.45 (s, 1H), 7.88 (s, 1H), 7.12-7.05 (m, 2H), 6.97-6.91 (m, 2H), 6.10 (t, J=6.4 Hz, 1H), 5.27 (s, 1H), 5.14 (s, 1H), 4.23 (dt, J=6.3, 3.7 Hz, 1H), 3.80 (q, J=3.5 Hz, 1H), 3.64 (d, J=12.0 Hz, 1H), 3.57 (d, J=12.3 Hz, 1H), 2.16 (ddd, J=13.2, 6.1, 3.8 Hz, 1H), 2.03 (dt, J=13.1, 6.5 Hz, 1H). AMM (ESI) calced. for C$_{15}$H$_{15}$N$_3$O$_4$S 333.08, found 356.07 (MNa+).

**tC 2′, 3′,5′-tri-O-(acetyl)-β-D-ribonucleoside.** 3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (200 mg, 0.92 mmol) was dissolved in dry acetonitrile (10 mL) under N$_2$. Bis(trimethylsilyl)acetamide (350 μL, 1.43 mmol) was added and the reaction mixture was heated to 78 °C. After 20 minutes at 78 °C the reaction was cooled
to room temperature and 1,2,3,5-tetra-acetyl-β-D-ribofuranose (300 mg, 0.94 mmol) was added followed by Trimethylsilyl trifluoromethanesulfonate (210 μL, 1.16 mmol) and the reaction was returned to 78 °C for 2.5 hours. The reaction mixture was poured into sodium bicarbonate solution (100mL, 5%), extracted with dichloromethane (2X 100mL) and evaporated. Purification via flash chromatography (EtOAc in cyclohexane) resulted in the desired product (380 mg, 0.80 mmol) 87% yield. $^1$HNMR (CDCl$_3$) δ 10.20 (s, 1H), 7.26 (s, 1H), 7.18 (dd, J=7.9,1.1 Hz, 1H), 6.95 (ddd, J=8.0, 6.6, 2.2 Hz, 1H), 6.80 (qd, J=7.7, 1.7 Hz, 2H), 6.05 (d, J=4.4 Hz, 1H), 5.34 (dd, J=5.7, 4.4 Hz, 1H), 5.26 (t, J=5.5 Hz, 1H), 4.01 (q, J=7.2 Hz, 1H), 2.11 (s, 3H), 2.00 (d, J=5.8 Hz, 6H), 1.15 (t, J=5.5 Hz, 1H), 4.01 (q, J=7.2 Hz, 1H), 2.11 (s, 3H), 2.00 (d, J=5.8 Hz, 6H), 1.15 (t, J=7.2 Hz, 2H).

tC-β-D-ribonucleoside. tC 2′, 3′,5′-tri-O-(acetyl)-β-D-ribonucleoside (340 mg, 0.715 mmol) was dissolved in methanol (3 mL) and a solution of 30 % sodium methoxide in methanol (20.3 μL, 0.109 mmol) was added and stirred at room temperature 18 hrs. The product was suspended in water (5mL) and filtered to yield (142 mg, 0.406 mmol) 57% yield. $^1$HNMR ((CD$_3$)$_2$SO) δ 10.43 (s, 1H), 7.96 (s, 1H), 7.07 (ddt, J=7.6, 3.2, 1.5 Hz, 2H), 6.96-6.88 (m, 2H), 5.72 (d, J=3.1Hz, 1H), 5.39 (d, J=4.5 Hz, 1H), 5.21 (t, J=5.0 Hz, 1H), 5.03 (d, J=4.9 Hz, 1H), 3.95 (q, J=3.9, 3.1 Hz, 2H), 3.83 (dt, J=5.6, 2.9 Hz, 1H), 3.70 (ddd, J=12.1, 5.0, 2.9 Hz, 1H), 3.56 (ddd, J=12.2, 5.0, 2.8 Hz, 1H).
5-(2-amino-4-methoxyphenyl)sulfanyl|pyrimidine-2,4(1H,3H)-dione. 2-((2-amino-4-methoxyphenyl)disulfanyl)-4-methoxynilne (3.1 g, 10.1 mmol) was placed in a dried 100 mL round-bottomed flask under N₂. Anhydrous diglyme (20 mL) was added with stirring, followed by the addition of water (90 μL, 5 mmol) and triethylphosphine (5.0 mL of a 1 M solution in THF). The reaction was allowed to stir for 30 minutes at room temperature to complete the reduction of the disulfide. Next, 5-bromouracil (3.85 g,
20.2 mmol) and anhydrous sodium carbonate (2.14 g, 20.2 mmol) were added and the reaction mixture was heated with stirring at 120 °C for 1 hour. The reaction mixture was allowed to cool to room temperature and the product, which was present as a white precipitate, was collected by filtration, washed with diglyme and then water, and then dried. NMR measurements showed it to be of high purity (2.3 g, 8.68 mmol) in 86% yield. $^1$HNMR ((CD$_3$)$_2$SO) δ 11.20 (s, 2H), 7.40 (s, 1H), 7.28 (d, J=8.5, 1H), 6.28 (d, J=2.7 Hz, 1H), 6.13 (dd, J=8.5, 2.7 Hz, 1H), 5.63 (s, 2H), 3.68 (s, 3H). $^{13}$CNMR ((CD$_3$)$_2$SO) δ 163.12, 161.47, 151.46, 151.46, 151.11, 143.11, 137.73, 107.15, 105.45, 103.32, 99.06, 54.84. AMM(ESI) calcd. for C$_{11}$H$_{11}$N$_3$O$_3$S 265.02, found 266.06 (MH+).

**8-methoxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one.** 5-[(2-amino-4-methoxyphenyl)sulfanyl]pyrimidine-2,4(1H,3H)-dione (2.07 g, 7.8 mmol) was suspended in 1-butanol (40 mL) under nitrogen and 37 % hydrochloric acid (8 mL) was added. The reaction mixture was heated at 120 °C for 3 days with stirring. The reaction mixture was then allowed to cool to 4 °C in a refrigerator, resulting in the precipitation of the product, which was collected by filtration. This crude product was added to a 5% aqueous solution of ammonium hydroxide at 50 °C and stirred, resulting in recrystallization that produced the product as a yellow precipitate. The product was collected by filtration, washed with water, and allowed to dry resulting in (1.66 g, 6.7 mmol) 86% yield. $^1$HNMR ((CD$_3$)$_2$SO) δ 10.18 (s, 1H), 7.43 (s, 1H), 6.98 (d, J=8.5, 1H), 6.60 (d, J=2.6 Hz, 1H), 6.55 (dd, J=8.6,
2.7 Hz, 1H), 3.70 (s, 3H). AMM (ESI) calcd. for \( \text{C}_{11}\text{H}_{9}\text{N}_{3}\text{O}_{2}\text{S} \) 247.04, found 495.09 (2MH+).

8-methoxy-3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside. 8-methoxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (400 mg, 1.60 mmol) was suspended in dry acetonitrile (8 mL) in a Schlenk tube under \( \text{N}_2 \). Bis(trimethylsilyl)acetamide (449 μL, 1.84 mmol) was added and the reaction mixture allowed to stir at 50 °C for 1 hour until the starting material had completely dissolved. The reaction mixture was allowed to cool to room temperature and 3′,5′-di-O-(p-toluoyl)-2′-deoxy-α-D-ribofuranosyl chloride (684 mg, 1.76 mmol) was added with stirring. The reaction mixture was cooled to 0 °C and tin(IV) chloride (37 μL, 0.32 mmol) was added dropwise. The reaction mixture was stirred at 0 °C, then allowed to warm to room temperature, and the progress was tracked by thin layer chromatography. The reaction was complete in 45 minutes. At this time, the mixture was diluted with ethyl acetate (40 mL), washed with a sodium bicarbonate, and dried. Rotary evaporation followed by purification using flash chromatography (EtOAc in cyclohexane) yielded the pure β anomer of the product as a yellow solid. (355 mg, 0.656 mmol) 41 % yield. \(^1\)HNMR (CDCl₃) δ 8.52 (s, 1H), 7.99-7.81 (m, 4H), 7.44 (s, 1H), 7.29-7.26 (m, 2H), 7.23-7.17 (m, 2H), 6.81 (d, J=8.5, 1H), 6.64 (d, J=2.5 Hz, 1H), 6.52 (dd, J=8.6, 2.6 Hz, 1H), 6.36 (dd, J=8.2,5.5 Hz, 1H), 5.59 (dt, J=6.5, 1.8 Hz, 1H), 4.77 (dd, J=12.3, 3.0 Hz, 1H), 4.68 (dd, J=12.3, 3.2 Hz, 1H), 4.60-4.55 (m, 1H), 3.77 (s,
3H), 2.92 (ddd, J=14.5, 5.6, 1.6 Hz, 1H), 2.43 (s, 3H), 2.37 (s, 3H), 2.22 (ddd, J=14.6, 8.2, 6.4 Hz, 1H). \textsuperscript{13}CNMR (CDCl\textsubscript{3}) \(\delta\) 166.11, 166.03, 161.00, 159.48, 154.47, 144.44, 144.32, 136.75, 132.92, 129.78, 129.54, 129.41, 129.23, 126.52, 126.42, 126.36, 110.88, 106.92, 104.26, 97.83, 87.00, 83.45, 75.19, 64.08, 55.64, 39.21, 21.70.

\textbf{8-methoxy-tC 2'-deoxy-\beta-D-ribonucleoside.} 8-methoxy-tC 3',5'-di-O-(p-toluoyl)-2'-deoxy-\beta-D-ribonucleoside (103 mg, 0.172 mmol) was dissolved in methanol (5 mL) and a solution of 30% sodium methoxide in methanol (96 \(\mu\)L, 0.517 mmol) was added dropwise. The reaction progress was followed by thin layer chromatography and found to be complete in 30 min. The reaction was quenched by the addition of acetic acid (50 \(\mu\)L, 0.872 mmol), and the solvent was removed by rotary evaporation. Purification by flash chromatography (CH\textsubscript{3}OH in CH\textsubscript{2}Cl\textsubscript{2}) yielded the product as a yellow solid (60 mg, 0.165 mmol) in 96% yield. \textsuperscript{1}HNMR ((CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 10.38 (s, 1H), 7.89 (s, 1H), 7.01 (d, J=8.5, 1H), 6.61 (d, J=2.6 Hz, 1H), 6.57 (dd, J=8.6, 2.7 Hz, 1H), 6.10 (t, J=6.4 Hz, 1H), 5.26 (s, 1H), 5.12 (s, 1H), 4.23 (dt, J=6.5, 3.6 Hz, 1H), 3.80 (q, J=3.5 Hz, 1H), 3.70 (s, 3H), 3.63 (dd, J=11.9, 3.6 Hz, 1H), 3.57 (dd, J=12.0, 3.6, 1H) 2.16 (ddd, J=13.2, 6.1, 3.7 Hz, 1H), 2.03 (dt, J=13.1, 6.5 Hz, 1H). \textsuperscript{13}CNMR ((CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 159.63, 158.80, 153.75, 137.51, 135.42, 126.79, 109.32, 106.53, 103.37, 94.70, 87.46, 85.39, 69.82, 60.83, 55.23, 48.59. AMM(ESI) calcd. for C\textsubscript{16}H\textsubscript{17}N\textsubscript{3}O\textsubscript{5}S 363.09, found 727.19 (2MH+).
2-[(2-amino-5-methoxyphenyl)disulfanyl]-5-methoxyaniline. 6-Methoxy-2-methylbenzothiazole (1.07 g, 6.0 mmol) was placed in a dried 50 mL side-armed flask under N₂. Hydrazine hydrate (5 mL) was added and the reaction mixture was heated at 100 °C for 17 hours with stirring. The mixture was allowed to cool to room temperature, diluted with water (25 mL), and the product sulfide was oxidized by the addition of 30% H₂O₂ (7 mL). This aqueous mixture was extracted with ethyl acetate (3 × 50 mL) and the resulting organic phase dried. Removal of the solvent by rotary evaporation yielded the product disulfide product as a yellow oil (0.55 g, 3.6 mmol) in 60 % yield. \(^1\)HNMR ((CD\(_3\)_2SO) \(\delta\) 6.80 (dd, \(J=8.8, 2.9\) Hz, 1H), 6.73 (d, \(J=8.8\) Hz, 1H), 6.62 (d, \(J=2.9, 2.6\) Hz, 1H), 3.55 (s, 3H).

5-[(2-amino-5-methoxyphenyl)sulfanyl]pyrimidine-2,4(1H,3H)-dione. 2-[(2-amino-5-methoxyphenyl)disulfanyl]-5-methoxyaniline (semi-crude from oxidative degradation, ~ 1.7 mmol) was placed in a dried 50 mL round-bottomed flask under N₂. Anhydrous diglyme (4 mL) was added with stirring, followed by the addition of water (22 \(\mu\)L, 1.2 mmol) and triethylphosphine (1.2 mL of a 1 M solution in THF). The reaction was allowed to stir for 30 minutes at room temperature to complete the reduction of the disulfide. Next, 5-bromouracil (0.83 g, 4.3 mmol) and anhydrous sodium carbonate (0.46 g, 4.3 mmol) were added and the reaction mixture was heated with stirring at 120 °C for 1.5 hours. Purification via silica gel flash chromatography (CH₃OH in CH₂Cl₂) resulted
in product as a dark-colored semi-crude solid. (~ 0.83 mmol), approximately 24 % yield.

$^1$HNMR ((CD$_3$)$_2$SO) $\delta$ 11.38 (s, 2H), 7.61 (s, 1H), 6.91 (d, J=2.9, 1H), 6.74 (dd, J=8.8, 2.9 Hz, 1H), 6.67 (d, J=8.8 Hz, 1H), 5.07(s, 2H), 3.64 (s, 3H).

7-methoxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one. 5-[(2-amino-5-methoxyphenyl)sulfanyl]pyrimidine-2,4(1H,3H)-dione (~ 0.83 mmol), semi-crude from prior step) was suspended in 1-butanol (10 mL) under N$_2$ and 37 % hydrochloric acid (1 mL) was added. The reaction mixture was heated at 120 °C under nitrogen with stirring for 1 day. The solvent and acid were then removed by rotary evaporation, and the product was purified by flash chromatography (CH$_3$OH in CH$_2$Cl$_2$) to yield a yellow solid (55 mg, 0.22 mmol) 27 % yield. $^1$HNMR ((CD$_3$)$_2$SO) $\delta$ 11.86-11.36 (m, 1H), 7.57 (s, 1H), 6.91 (d, J=8.6, 1H), 6.75 (d, J=2.6 Hz, 1H), 6.71 (dd, J=8.6, 2.8 Hz, 1H), 3.71 (s, 3H).

AMM (ESI) calcd. for C$_{11}$H$_9$N$_3$O$_2$S 247.04, found 495.09 (2MH+).

7-methoxy-tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside. 7-methoxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (35 mg, 0.14 mmol) was suspended in dry acetonitrile (1 mL) in a Schlenk tube under N$_2$. Bis(trimethylsilyl)acetamide (40 μL, 0.163 mmol) was added and the reaction mixture allowed to stir at 50 °C for 1 h. The reaction mixture was allowed to cool to room temperature and 3′,5′-di-O-(p-toluoyl)-2′-
deoxy-α-D-ribofuranosyl chloride (61 mg, 0.156 mmol) was added with stirring. The reaction mixture was cooled to 0 °C and tin(IV) chloride (3.3 μL, 0.028 mmol) was added dropwise. The reaction mixture was stirred at 0 °C, then allowed to warm to room temperature, and the progress was tracked by thin layer chromatography. The reaction was complete in 90 min. At this time, the mixture was diluted with ethyl acetate (15 mL), washed with a sodium bicarbonate, and dried. Rotary evaporation followed by purification using flash chromatography (EtOAc in cyclohexane) yielded the pure β anomer of the product as a yellow solid. (10 mg, 0.017 mmol) 12 % yield.

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\begin{align*}
\text{HNMR} & \quad (\text{CDCl}_3) \delta 9.02 (s, 1H), 7.98-7.88 (m, 4H), 7.37 (s, 1H), 7.27 (t, J=1.3 \text{ Hz}, 2H), 7.24-7.20 (m, 2H), 7.00 (d, J=8.8, 1H), 6.62 (dd, J=8.8, 2.8 \text{ Hz}, 1H), 6.45 (d, J=2.7 \text{ Hz}, 1H), 6.38 (dd, J=8.3, 5.5 \text{ Hz}, 1H), 5.59 (dt, J=6.6, 1.7 \text{ Hz}, 1H), 4.77 (dd, J=12.3, 3.0 \text{ Hz}, 1H), 4.67 (dd, J=12.3, 3.3 \text{ Hz}, 1H), 4.58 (q, J=2.8 \text{ Hz}, 1H), 3.74 (s, 3H), 2.92 (ddd, J=14.5, 5.6, 1.6 \text{ Hz}, 1H), 2.43 (s, 3H), 2.37 (s, 3H), 2.25-2.18 (m, 1H). \\
\text{CNMR} & \quad (\text{CDCl}_3) \delta 166.39, 166.36, 156.88, 154.64, 144.72, 144.64, 132.98, 130.09, 129.81, 129.69, 129.50, 129.06, 126.70, 126.59, 118.91, 118.08, 112.80, 111.89, 97.18, 87.23, 83.74, 75.54, 64.43, 55.86, 39.43, 29.94, 21.98.
\end{align*}
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7-methoxy-tC 2′-deoxy-β-D-ribonucleoside. 7-methoxy-tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside (8.1 mg, 0.014 mmol) was dissolved in methanol (0.5 mL) and a solution of 30 % sodium methoxide in methanol (8.4 μL, 0.045 mmol) was added.
dropwise. The reaction progress was followed by thin layer chromatography and found to be complete in 30 min. The reaction was quenched by the addition of acetic acid (10 μL, 0.17 mmol) and the solvent was removed by rotary evaporation. Purification by flash chromatography (CH$_3$OH in CH$_2$Cl$_2$) yielded the product as a yellow solid (3.4 mg, 0.0097 mmol) 69 % yield. $^1$HNMR (CD$_3$OD) δ 7.91 (s, 1H), 6.85-6.80 (m, 1H), 6.68 (dd, J=8.8, 2.7 Hz, 1H), 6.63 (d, J= 2.7 Hz, 1H), 6.21 (t, J=6.4 Hz, 1H), 4.39 (dt, J=6.4, 3.9 Hz, 1H), 3.95 (q, J=3.5 Hz, 1H), 3.87-3.77 (m, 2H), 3.72 (s, 3H), 2.38 (ddd, J=13.6, 6.2, 4.0 Hz, 1H), 2.17 (dt, J=13.3, 6.5 Hz, 1H) 13CNMR ((CD$_3$)$_2$SO) δ 158.27, 157.45, 145.59, 135.79, 130.38, 119.14, 118.96, 113.83, 112.46, 98.31, 88.98, 87.78, 71.71, 62.48, 56.07, 42.17. AMM (ESI) calcd. for C$_{16}$H$_{17}$N$_3$O$_5$S 363.09, found 727.18 (2MH+).

8-hydroxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one. 8-methoxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (20 mg, 0.081 mmol) was dissolved in dry dichloromethane (1.3 mL) under N$_2$ and cooled in a dry ice acetone bath. Boron tribromide 1M in dichloromethane (243 μL, 0.243 mmol) was added dropwise to the reaction and stirred at -78 °C 5.25 hours and then kept at -20 °C for 11 hours. Dilution with ethyl acetate and evaporation followed by purification via silica gel flash chromatography (CH$_3$OH in CHCl$_3$) led to desired product (8.3 mg, 0.035 mmol) in 51% yield. $^1$HNMR (CD$_3$OD) δ 7.25 (s, 1H), 6.80 (d, J=8.3 Hz, 1H), 6.45-6.39 (m, 2H).
8-hydroxy-tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside. 8-hydroxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (19 mg, 0.031 mmol) was dissolved in dry acetonitrile (0.5 mL) under N₂. Bis(trimethylsilyl)acetamide (30 μL, 0.0125 mmol) was added and the reaction mixture was stirred at room temperature. After 55 minutes 3′,5′-di-O-(p-toluoyl)-2′-deoxy-α-D-ribofuranosyl chloride (44 mg, 0.113 mmol) was added and the reaction cooled to 0 °C. Tin (IV) chloride (drops) in acetonitrile (350 μL) was added to the reaction and stirred at 0. Additional bis(trimethylsilyl)acetamide (30 μL, 0.0125 mmol) was added two times and the reaction moved between 0 °C and -20 °C over 4 days. Dilution with ethyl acetate (10mL), washing with sodium bicarbonate and brine, drying and evaporation were performed. Purification via flash chromatography (CH₃OH in CHCl₃) resulted in the desired product (6.6 mg, .012 mmol) 15% yield.

8-hydroxy-tC-2′-deoxy-β-D-ribonucleoside. 8-hydroxy-tC 3′,5′-di-O-(p-toluoyl)-2′-deoxy-β-D-ribonucleoside (6.6 mg, 0.012 mmol) was dissolved in methanol (0.6 mL) under N₂. Sodium methoxide 30% solution in methanol (10 μL, 0.050 mmol) was added and the reaction stirred overnight in the dark. Additional sodium methoxide 30% solution
in methanol (10 μL, 0.050 mmol) was added to the reaction and when no starting material remained via thin layer chromatography, acetic acid (6 μL, 0.10 mmol) was added to the reaction and it was evaporated. Purification via flash chromatography (CH₃OH in CH₂Cl₂) resulted in product (3.5 mg, 0.010 mmol) 80% yield.

8-trifluromethanesulfonate-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one.

8-hydroxy-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (9 mg, 0.039 mmol) was dissolved in dry dichloromethane (1mL) under N₂ and cooled to 0 °C. Triethylamine (6.5 μL, 0.047mmol) followed by trifluoromethanesulfonic anhydride (7.2 μL, 0.043 mmol) were added dropwise to the reaction and the mixture was allowed to warm to room temperature. After 2 hours, the reaction was cooled back to 0 °C and 2M hydrochloric acid (40 μL, 0.078 mmol) was added and the reaction was allowed to warm to room temperature.

1-diethylaminonaphtalene. Potassium tert-butoxide (308 mg, 2.75 mmol) was dissolved in dry tetrahydrofuran (1mL) under N₂. Diethylamine ( 60 μL, 0.55 mmol) was added followed by 1-chloronapthalene (70 μL, 0.5 mmol) and allyl[1,3-bis(2,6-diisopropylphenyl)-imidazol-2-ylidene]palladium (II) chloride (15 mg, 0.026 mmol) and stirred at room temperature. Water was added to the reaction and the organics were
extracted with diethyl ether, dried, filtered and evaporated. Purification via flash chromatography (CH₂Cl₂ in Cyclohexanes) resulted in product (0.4 mg, 0.0021 mmol) 0.4% yield.

2-methyl-1,3-benzothiazol-6-diethylamine. Acetaldehyde (562 μL, 10 mmol) was dissolved in acetonitrile (10 mL) under N₂. 2-methyl-1,3-benzothiazol-6-amine (164 mg, 1 mmol) was combined with sodium cyanoborohydride (94 mg, 1.5 mmol) in acetonitrile (10 mL) and added to the above reaction followed by acetic acid (1 mL). After 30 minutes at room temperature the reaction was diluted with methanol and evaporated. The residue was suspended between ethyl acetate and water, dried and evaporated. Purification using flash silica gel chromatography (Ethyl Acetate in Hexanes) resulted in the product (151 mg, 0.69 mmol) 69% yield. ¹HNMR (CDCl₃) δ 7.70 (dd, J=9.0, 0.4 Hz, 1H), 6.95 (d, J=2.4 Hz, 1H), 6.80 (dd, J=9.0, 2.6 Hz, 1H), 2.68 (s, 3H), 1.19 (t, J=7.2 Hz, 4H), 1.12 (t, J=7.1 Hz, 6H).

5-[(2-amino-5-diethylaminophenyl)sulfanyl]pyrimidine-2,4(1H,3H)-dione. 2-methyl-1,3-benzothiazol-6-diethylamine (220 mg, 1 mmol) was dissolved in hydrazine (2.2 mL) under N₂. This was refluxed 18 hours and then 5-bromouracil (142 mg, 0.75 mmol) and sodium carbonate (79 mg, 0.75 mmol) were added and the reaction refluxed for 30 minutes. Additional 5-bromouracil (142 mg, 0.75 mmol) and sodium carbonate
(79 mg, 0.75 mmol) were added and the reaction continued to reflux. Dilution with water and then evaporation. Purification via flash silica gel chromatography (CH$_3$OH in CH$_2$Cl$_2$) led to the possible product.
Oxygen Tricyclic Cytosine Analogs

A first attempt to synthesize the nucleobase of tC₀ under the same conditions as the nucleoside was synthesized in the literature in the lab, lead to the creation of a new compound (figure 2-0-9).³ In the unsubstituted nucleoside synthesis, mesitylsulfonylation occurs at O⁴. This mesitylsulfonyl ester is poised for substitution at the C4 carbon by the amine nitrogen of the aminophenol under basic conditions. Crystallization of the resulting unknown compound was achieved using slow evaporation and solving the crystal structure revealed that the 2-mesitylenesulfonyl chloride had reacted with the N1 of the 5-bromouracil instead of the C4 carbonyl (figure 2-0-10). This N-Mes-bromouracil was re-subjected to the above reaction conditions as the N1 was now protected from reaction and reactivity remained possible for the O⁴ oxygen. The compound did not appear to react with the aminophenol in this case either.
The evidenced greater reactivity of the N1 position on 5-bromouracil was harnessed through the use of a boc protecting group (figure 2-0-11). It has been previously shown that it was possible to place this group at the N1 position without also protecting the N3 nitrogen. The resonance form of DMAP which has a negative charge on the pyrimidinal nitrogen utilizes these electrons to attack the carbonyl carbon of boc anhydride. Upon reformation of the carbon oxygen double bond carbonate is released which can pick up hydrogen from N1. 5-Bromouracil then replaces the DMAP at the carbonyl carbon of the BOC group via nucleophilic attack by the N1 nitrogen.

This boc protected 5-bromouracil was reacted under the same standard tCo synthesis conditions previously described (figure 2-0-2). This reaction did not result in the desired formation of the secondary amine at the C4 carbon of 5-bromouracil. There was NMR evidence that the boc protecting group was removed during the course of the reaction, which likely resulted in the undesirable side reaction of the 2-
mesitylenesulfonyl chloride with the N1 nitrogen as was seen in the reaction of the unprotected compound.\textsuperscript{86} After the Appel chemistry for activation of carbonyls had been used successfully to synthesize tC\textsuperscript{9} nucleoside secondary amines in the laboratory, as will be discussed in Chapter 3 Section 1, these conditions were tried to form the amine bond between 5-bromouracil and aminophenol en route to the tC\textsuperscript{9} nucleobase (figure 2-0-12).\textsuperscript{87} This chemistry had been successful in the synthesis of nucleoside analogs related to the oxygen tricyclic cytosine parent.\textsuperscript{88} Triphenyl phosphine activation was also seen by the authors who synthesized the parent compound.\textsuperscript{89} The phosphorous of triphenyl phosphine uses its lone pair of electrons to extract chlorine from carbon tetrachloride creating the trichloride carbanion. These anionic electrons deprotonate N3 and this nitrogen can form a double bond with the C4 carbonyl forcing the double bond electrons onto the oxygen and causing an oxyanion. The oxyanion attacks the positively charged phosphorous and releases the chlorine anion. This chlorine can then substitute for the oxygen at the C4 carbon of 5-bromouracil. Upon addition of the aminophenol with DBU, the amine substitutes for this chlorine at C4 resulting in the desired product. This reaction resulted in trace amounts of the desired secondary amine formation with concomitant removal of the boc protecting group as detectable by mass spectroscopy.
Repeating this reaction led to confirmation of the product by NMR but isolation was difficult. Following multiple chromatographic attempts to remove the triphenylphosphine oxide product, it was only possible to recover trace amounts of the secondary amine. Following optimization of chromatography conditions for the removal of this impurity the deprotected secondary amine of the tC⁰ nucleobase was recoverable in an isolated yield of 27% from these reaction conditions.
An ethyl carbomate protecting group was installed at the N1 position in place of the boc protecting group to test the liability of this group under the reaction conditions. This reaction occurs with ethyl chloroformate in dichloromethane with assistance from potassium carbonate (figure 2-0-13). As in the reaction with the boc anhydride, the deprotonated N1 nitrogen can attack the carbonyl carbon, this time of the acyl chloride and upon reformation of the carbon oxygen double bond a chlorine anion is expelled. This ethyl carbomate protecting group installed at the N1 position of 5-bromouracil is also labile to the Appel reaction conditions (figure 2-0-5). Secondary amine tC nucleobase is evidenced in the NMR when aminophenol is reacted with ethyl carbomate protected 5-bromouracil under these conditions, but was difficult to effectively isolate from the triphenyl phosphine oxide.

A benzyl protecting group can be alternatively installed at the N1 position through a combination of hexamethyldisilazane and trimethylsilyl-chloride in dichloroethane
followed by benzyl bromide and iodine (figure 2-0-14).\textsuperscript{90} Without the presence of carbonyl functionality this protecting group may have been less reactive under the secondary amine formation conditions. The HMDS and TMSCl mixture silylated the C2 carbonyl oxygen with the assistance of the lone pair of electrons on the N1 nitrogen. Upon addition of the iodine, the silyl group is removed and the assisting electrons of the N1 are free to substitute for the bromine on the benzyl bromide. This protecting group remained intact under the reaction conditions and allowed for recovery of the benzyl protected secondary amine of the parent tC\textsuperscript{6} nucleobase in decent yields. Below is presented a table (Table 1) of reaction conditions for synthesis of this unsubstituted and the substituted nucleobase secondary amines.
Table 1: Synthesis of nucleobase secondary amines. Analog is the name of the substituted product referring to figure 2-4.19. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Protecting</th>
<th>Activator</th>
<th>Solvent</th>
<th>Temp.</th>
<th>Activation</th>
<th>Substitution</th>
<th>Scale</th>
<th>Yield</th>
<th>MS</th>
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<td>DCM/CCl4</td>
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<td>5hrs</td>
<td>15minat0</td>
<td>1g</td>
<td>NA</td>
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<tr>
<td>7-Cl</td>
<td>Benzyl</td>
<td>MesCl</td>
<td>DCM</td>
<td>40</td>
<td>overnight</td>
<td>2.5hrs rt</td>
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<td>6</td>
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<tr>
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<td>Benzyl</td>
<td>MesCl</td>
<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
<td>500mg</td>
<td>30</td>
<td>Yes</td>
</tr>
<tr>
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<td>MesCl</td>
<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
<td>2g</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>8-Cl</td>
<td>Benzyl</td>
<td>PPh3</td>
<td>DCM/CCl4</td>
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<td>4hrs</td>
<td>1hrat44</td>
<td>1g</td>
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<td>Benzyl</td>
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<td>DCM/CCl4</td>
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<td>6hrs</td>
<td>44</td>
<td>5g</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>8-Cl</td>
<td>Benzyl</td>
<td>PPh3</td>
<td>DCM/CCl4</td>
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<td>5hrs</td>
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<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
<td>2g</td>
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<td>TIBSCI</td>
<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td></td>
<td>50mg</td>
<td>NA</td>
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</tr>
<tr>
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<td>Boc</td>
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<td>ACN/PYR</td>
<td>-10</td>
<td>to warm</td>
<td>4hrrt/overnight</td>
<td>0</td>
<td>Deprot.</td>
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</tr>
<tr>
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<td>MesCl</td>
<td>DCM</td>
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<td>overnight</td>
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<td>25</td>
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<tr>
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<td>Benzyl</td>
<td>MesCl</td>
<td>DCM</td>
<td>40</td>
<td>Overnight</td>
<td>7hrs rt</td>
<td>500mg</td>
<td>&lt;20</td>
<td></td>
</tr>
<tr>
<td>8-F</td>
<td>Benzyl</td>
<td>MesCl</td>
<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
<td>2g</td>
<td>NA</td>
<td></td>
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<tr>
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<td>Ethyl Carb</td>
<td>POCl3</td>
<td>ACN/PYR</td>
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<td>to warm</td>
<td>overnight</td>
<td>100mg</td>
<td>Deprot.</td>
<td></td>
</tr>
<tr>
<td>8-Ome</td>
<td>Benzyl</td>
<td>PPh3</td>
<td>DCM/CCl4</td>
<td>44</td>
<td>5hrs</td>
<td>at0</td>
<td>100mg</td>
<td>NA</td>
<td></td>
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</table>
Table 1 continued: Synthesis of nucleobase secondary amines. Analog is the name of the substituted product referring to figure 2-0-19. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Protecting</th>
<th>Activator</th>
<th>Solvent</th>
<th>Activatel.</th>
<th>Temp.</th>
<th>Temp.</th>
<th>Scale</th>
<th>MS</th>
<th>Yield</th>
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<tr>
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<td>Benzyl</td>
<td>PPh3</td>
<td>DCM/CCl4</td>
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<td>44</td>
<td>200mg</td>
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<td>DCM/CCl4</td>
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<td>44</td>
<td>44</td>
<td>3.3g</td>
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<td>44</td>
<td>44</td>
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<td>NA</td>
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<td>0</td>
</tr>
<tr>
<td>8-Ome</td>
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<td>MesCl</td>
<td>DCM</td>
<td>overnight</td>
<td>NA</td>
<td>5mg</td>
<td>200mg</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8-Ome</td>
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<td>DCM</td>
<td>3hrs</td>
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<td>overnight</td>
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<td>rt</td>
<td>1.25hrs</td>
<td>50mg</td>
<td>deprotected</td>
</tr>
<tr>
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<td>Boc</td>
<td>ACN/PYR</td>
<td>overnight</td>
<td>NA</td>
<td>overnight</td>
<td>100mg</td>
<td>50mg</td>
<td>deprotected</td>
</tr>
<tr>
<td>8-Ome</td>
<td>Benzyl</td>
<td>Boc</td>
<td>ACN/PYR</td>
<td>overnight</td>
<td>NA</td>
<td>overnight</td>
<td>50mg</td>
<td>50mg</td>
<td>deprotected</td>
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<td>8-Ome</td>
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<td>Boc</td>
<td>ACN/PYR</td>
<td>overnight</td>
<td>NA</td>
<td>overnight</td>
<td>100mg</td>
<td>50mg</td>
<td>deprotected</td>
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<tr>
<td>8-Ome</td>
<td>Benzyl</td>
<td>Boc</td>
<td>ACN/PYR</td>
<td>overnight</td>
<td>NA</td>
<td>overnight</td>
<td>to warm</td>
<td>100mg</td>
<td>deprotected</td>
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Note: Some isolated reactions or those for purely production purposes are excluded from this table.
Table 1 continued: Synthesis of nucleobase secondary amines. Analog is the name of the substituted product referring to figure 2a-19. Some yields are approximations based on NMR and in the event that no yield is given, isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Protecting</th>
<th>Activator</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Time Activation</th>
<th>Time Substitution</th>
<th>Scale</th>
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<tbody>
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<td>Benzyl</td>
<td>PPh3</td>
<td>DCM/CCl4</td>
<td>44</td>
<td>5hrs</td>
<td>40minat0</td>
<td>1g</td>
<td>0.20%</td>
</tr>
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<td>DCM/CCl4</td>
<td>44</td>
<td>7hrs</td>
<td>2hrsat0</td>
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<td>31%</td>
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<tr>
<td>Tet</td>
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<td>BOP</td>
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<td>Same time</td>
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<td>60%</td>
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<tr>
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<td>PYBOP</td>
<td>THF</td>
<td>60</td>
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<tr>
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<td>MesCl</td>
<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
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</tr>
<tr>
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<td>DCM</td>
<td>rt</td>
<td>overnight</td>
<td>rt</td>
<td>500mg</td>
<td>22%</td>
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</table>
Some additional conditions were attempted to activate the C4 carbonyl for attack by the amine on the aminophenol. Reacting protected 5-bromouracil with phosphorous oxychloride and n-methyl imidazole in acetonitrile creates the same aryl chloride intermediate as the Appel conditions (figure 2-0-15).\(^{91}\) These conditions had been successful in the synthesis of some related compounds.\(^{92,93}\) NMI functions as a nucleophile to activate phosphorous oxychloride through the formation of a dioxophosphonium complex.\(^{93}\) This complex accepts the oxygen electrons from the 5-bromouracil carbonyl oxygen at C4 as well as provides the base for the deprotonation of N3. The chlorine anion can substitute at C4 and results in an intermediate that can be substituted by the aminophenol. While these conditions are designed to give the same intermediate, the desired substitution product was not obtained when attempted with the boc protected 5-bromouracil. Deprotection of the starting material likely resulted in lack of formation or lack of reactivity of the aryl chloride.
Forming an intermediate in which a triazole was attached at C4 was another pathway to the tC<sup>0</sup> secondary amine nucleobase that was attempted (figure 2-0-16).<sup>70</sup>

Similarly to the conditions described above a dioxophosphonium complex is formed as a result of the triazole reaction with phosphorous oxychloride. This complex did not react with the benzyl protected 5-bromouracil. Initial reaction was tried in acetonitrile at room temperature and no reaction was seen. A solvent was selected with which the starting material was more soluble, but in DMF no reaction was observed even when the reaction was heated to 40 degrees.

Attempts to react the unprotected 5-bromouracil under the Appel conditions did not result in the recovery of any desired product. This prompted an NMR investigation of the reaction conditions. In this experiment it was revealed that there was little reaction with the aminophenol. Lack of substitution reactivity was attributed to a lack of activation and therefore assumed to be the result of little reaction between the starting material and the triphenylphosphine. A second NMR investigation of the reaction between the 5-bromouracil and the activated triphenylphosphine showed reaction to only a small extent resulting in mostly starting material.

Using the benzyl protected tC<sup>0</sup> secondary amine that had been synthesized, nucleophilic aromatic substitution to form the fluorescent nucleobase was attempted. This
reaction was performed using potassium fluoride in refluxing ethanol for days (figure 2-0-17). The alcohol attached to C1’ is in the position for nucleophilic aromatic substitution at C5 with the bromine as the leaving group. Under these conditions it was possible to isolate a small amount of fluorescent product and by NMR this chromophore appeared to be the desired tricyclic nucleobase which had been debenzylated during the course of the reaction. The tC° secondary amine that had lost its protecting group during the previous synthetic step was placed in a higher boiling solvent with a more dissociative fluorine source to encourage the reaction. After heating at 120 °C degrees for 4 hours in NMP with cesium fluoride the deprotected tC° secondary amine remained unreacted. This lack of reactivity may have been due to the contamination of secondary amine with triphenyl phosphine oxide. This triphenyl phosphine oxide may have been involved in undesirable side reactions.

![Diagram of nucleobase ring closure](image.png)

**Figure 2-0-17: Nucleobase ring closure.** Synthetic conditions presented for the formation of the ether and closure of the nucleobase to the desired tricyclic structure. For reaction success and failure see Table 2.
8-F-tC\textsuperscript{o} was the first substituted nucleobase analog upon which synthesis began using 4-fluoroaminophenol in the place of aminophenol in the reaction conditions (figure 2-0-18). Boc protected 5-bromouracil was reacted under the phosphorous oxychloride conditions and the reaction mixture was purified chromatographically with chlorinated solvents in attempts to isolate the secondary amine product (figure 2-0-15, 2-0-19). There was evidence that the nucleobase was deprotected under these conditions, and no desired product was recovered. Benzyl protected 5-bromouracil was reacted with the MesCl conditions and this resulted in a 25% yield of the desired product on a 50mg scale (figures 2-0-2, 2-0-19). Unfortunately scaling up of this reaction to the 500mg or the 2g scale resulted in decreased yields and increased difficulty in the isolation of the desired product from the side products. Side reactions likely were the result of debenzylation of the nucleobase starting material.
Ring closure of the isolated benzyl protected 8-F-tC\(^{\circ}\) nucleobase was achieved by refluxing in ethanol for 2 days with cesium fluoride (figure 2-0-17). It took scaling up of the reaction to isolate a mixture which contained enough product to be confirmed by mass spectroscopy. Side products from the debenzylation of the starting material were again the major source of contamination. This reaction took only one day, which is the same reaction time that was needed to observe product formation if the solvent is switched to butanol and the temperature increased to 101 °C. The yield of product from these higher temperature conditions was greater, which may also be attributable to the greater scale.

4-methoxy-2-amino phenol was reacted with ethyl carbamate protected 5-bromouracil under the POCl\(_3\) conditions in initial attempts to synthesize the 8-OMe-tC\(^{\circ}\) nucleobase secondary amine (figures 2-0-15, 2-0-18, 2-0-19). Deprotection of the nucleobase starting material was the only activity that could be confirmed in the reaction, and none of the desired product was recovered. Using the Appel chemistry conditions to couple the 4-methoxy-amino phenol to the benzyl protected 5-bromouracil it was possible to observe NMR evidence of the formation of the desired product, but the secondary amine was not recoverable (figures 2-0-12, 2-0-19). Doubling the scale of the reaction to 200mg led to production of the desired product in roughly 50% yield. However
performing the reaction on a gram scale made it prohibitively difficult to isolate the desired secondary amine product from the triphenyl phosphine oxide. Reaction was attempted under the MesCl conditions to eliminate the potential for triphenylphosphine oxide contamination, and this resulted in a comparable yield on the 50mg scale (figure 2-0-2, 2-0-19). Scaling up by 10 times however resulted in a 50% decrease in the isolable yield of the desired product due to the difficulty in separation from the unreacted starting materials.

Benzyl protected 8-OMe-tC₉ nucleobase was synthesized in trace amounts as detected by NMR upon heating the secondary amine produced under the above conditions to 101 °C in butanol for one day with cesium fluoride (figure 2-0-17). These higher temperature conditions with an alternative fluorine source were more successful than refluxing 3 days in ethanol with potassium fluoride. Those literature conditions resulted in the recovery of the remnants of the products of debenzylation of the starting material. A small scale reaction with cesium fluoride and refluxing in ethanol did not result in the desired nucleophilic aromatic substitution either.

Under Appel conditions, the 8-Cl-tC₉ secondary amine was synthesized in 18% yield on a one gram scale from benzyl protected 5-bromouracil and 4-chloro-aminophenol (figure 2-0-5, 2-0-19). Yet, on a 5 gram scale only 1.8% yield of isolable product was possible from a mixture with large amounts of triphenyl phosphine oxide. In a follow-up smaller scale reaction, the desired product was also inseparable from triphenylphosphine oxide, so the MesCl conditions were revisited. It was possible to obtain the desired secondary amine from the mesitylene intermediate in 22% yield on a
50mg scale (figure 2-0-2, 2-0-19). Changing the temperature of the carbonyl activation step from 40 degrees to room temperature resulted in double the yield of the desired product. This could possibly be attributed to the increase in scale of this reaction but an increase in reaction scale is ordinarily shown to have the opposite effect. Further increases in scale resulted in an increase in the debenzylation of the desired product and the debenzylated secondary amine was unable to be isolated.

Attempting to improve on the activation of the carbonyl carbon to substitution while avoiding the production of triphenylphosphine oxide, TIBSCl was substituted for MesCl. These conditions resulted in the formation of a small amount of the deprotected product (figure 2-0-20). In a similar manner to the 2-mesitylenesulfonyl chloride conditions, the oxygen of the C4 carbonyl attacks the silicon displacing the chlorine and priming the oxygen to be a better leaving group for when the aminophenol comes to substitute.

![Figure 2-0-20: TIBS activation of 5-bromouracil for amine formation. A related molecule was tried as an alternative to MesCl in the synthesis of secondary amines. Reaction was unsuccessful in generating product where R is a protecting group but did make some product where R is hydrogen.](image)

Refluxing in ethanol for four days with potassium fluoride led to NMR evidence of the benzyl protected 8-Cl-tC\(^0\) nucleobase in 15% yield (figure 2-0-17). A combination of calcium carbonate to increase the nucleophilicity of the 1’ oxygen for substitution and the more dissociative cesium fluoride in ethanol at 85 °C did not result in the same
product. Instead aminophenol was the main recovered material suggesting that these conditions were encouraging deamination of the starting material.\(^93\) A small scale reaction with just cesium fluoride in ethanol resulted in the recovery of the product of debenzylolation after 2 days. On a larger scale, these conditions resulted in the desired product which was recovered in comparable yield to the reaction where potassium was the counter ion. Using butanol as the solvent allowed reactions to be subjected to 101 °C, under these conditions it was again deamination that appeared to be the major reaction undergone by the starting material.

Starting material with the chlorine in a position para to the nitrogen was much more amenable to the formation of secondary amine 7-Cl-tC\(^\circ\) (figure 2-0-18, 2-0-19). Under triphenyl phosphine activation conditions, 1 gram of benzyl protected 5-bromouracil led to the desired product, which could not be isolated from the triphenyl phosphine oxide (figure 2-0-11). When using instead the 2-mesitylenesulfonyl chloride activator at 40 degrees, a small amount of the product was obtained (figure 2-0-2). In attempting to generate enough of the intermediate for the following synthetic methods to be carried out, the scale of the reaction was increased ten times, and this resulted in enough of the product to confirm its identity using mass spectrometry. There was difficulty in removing the side product from debenzylolation of the starting material. 7-Cl-tC\(^\circ\) was best obtained through activation at room temperature instead of at 40 degrees, and when these conditions were repeated on a 2 gram scale, it was possible to isolate the product with 51% yield.
Benzyl protected 7-Cl-tC\textsuperscript{o} secondary amine was refluxed in ethanol for three days with cesium fluoride and this may have resulted in some trace amounts of the nucleobase product as evidenced by NMR spectroscopy, but the major isolated product was that from the debenzylation of the starting material (figure 2-0-17). Using butanol as the solvent instead and heating at 120 °C led again to NMR evidence of possible product formation but this was not isolable from the starting material with which it was contaminated. 18-crown-6 was showing some utility in the laboratory for increasing the dissociation of the potassium fluoride through potassium sequestration (seen in Chapter 3 section 1). This combination of reactants was utilized in diglyme to attempt to ring close the 7-Cl-tC\textsuperscript{o} secondary amine at 120 degrees. The major result of this reaction turned out to be the deamination of the starting material.

Due to the difficulties in encouraging nucleophilic aromatic substitution with these substituted tricyclic cytosine nucleobase precursors, some alternative conditions were visited. One alternative ring closure method that was tried utilizes silver tetrafluoroborate instead of fluorine. Silver bonds to the bromine to aid it as a leaving group and weakly coordinating tetrafluoroborate anion might have been useful in the substitution at this position by the 1’ oxygen. This alternative reagent would likely have better solubility as well. 7-Cl-tC\textsuperscript{o} secondary amine was reacted with this reagent in refluxing ethanol, but these conditions led to no reaction of the starting material.
Copper iodide with 2-picolinic acid and potassium phosphate in DMSO at 90 °C was another ring closure procedure that was tried. The picolinic acid chelates the copper to free the iodine, Iodine anions can take the place of the fluorine anions in the original conditions to coordinate at the C5 position and assist in the leaving of the bromine. Potassium phosphate creates a basic environment which could assist in increasing the nucleophilicity of the phenolic oxygen through deprotonation, assisting the closure of the center ring of this tricycle through the formation of the oxygen (O1’) carbon (C5) bond. Benzyl protected 8-Cl-tC0 secondary amine was subjected to these conditions, but from purification all that was isolated were the remnants of starting material deamination (figure 2-0-21).

Ethyl carbomate protected 5-bromouracil was reacted with aminonaphthol under the alternative conditions using phosphorous oxychloride in attempting to synthesize tetC0 nucleobase secondary amine. These reaction conditions resulted in the removal of the ester protecting group from 5-bromouracil (figure 2-0-15). Benzyl protected 5-bromouracil was reacted under the Appel conditions with aminonaphthol resulting in NMR evidence of the secondary amine tetC0 product. The majority of the compound recovered was contaminated by triphenyl phosphine oxide (figure 2-0-12). On a smaller scale it was possible to isolate the desired secondary amine product in 31% yield.
Some alternative conditions were investigated to form the secondary amine of tetC without triphenylphosphine oxide, including activation with BOP in acetonitrile. DBU deprotonates the N3 nitrogen and this resonates to form a double bond with the C4 carbon forming the oxyanion. The oxyanion attacks the phosphorous of BOP, and the BOP oxygen substitutes for this 5-bromouracil oxyanion at C4 resulting in the formation of HMPA. The benzothiazole can assist with deprotonation of the aminophenolic nitrogen which needs deprotonation following substitution at C4 which displaced the benzotriazolyoxy group. Under these conditions, a compound was obtained in 60% yield which was possibly the desired secondary amine product (figure 2-0-22). The benzyl protected tetC secondary amine products isolated from different conditions all give NMR evidence of potential product formation, but the spectral data for the products vary between these different conditions.
PyBOP is preferential to BOP because it does not result in the formation of HMPA so this activator was tested as an alternative. PyBOP behaves in the same way as BOP described above, the only difference being that PyBOP has three pyrrolidines instead of three dimethyl amines attached to the phosphorous and thus does not product HMPA. Substituting this reagent did not result in the appearance of any of the desired product, even when using THF as the solvent and heating to 60 °C (figure 2-0-23). Using the MesCl conditions on a small scale it was possible to isolate the desired tetC\textsuperscript{5}\textsuperscript{o} secondary amine product in high yield (figure 2-0-2). Scaling up these promising conditions unfortunately resulted in a decrease in the yield of the product and a large amount of unreacted 5-bromouracil was difficult to remove from the desired product.

Triethylphosphine was tried as a substitute for triphenylphosphine and this resulted in a production of the deprotected product but in much worse yields than had been seen for the phenyl version. The following table (Table 2) displays the reaction conditions for the ring closure to form the oxygen tricyclic cytosine nucleobases.

Figure 2-0-23: PYBOB activation for tC\textsuperscript{5}\textsuperscript{o} secondary amine synthesis. An alternative peptide coupling reagent that does not generate HMPA was substituted in secondary amine formation synthesis. Reaction was unsuccessful.
Table 2: Ring closure of nucleobases. Analog is the name of the substituted product. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
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<th>Scale</th>
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Table 2 Continued: Ring closure of nucleobases Analog is the name of the substituted product. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

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<td>Butanol</td>
<td>CsF</td>
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</tr>
</tbody>
</table>
In one set of conditions tested calcium carbonate was combined with cesium fluoride and the benzyl protected tetC⁰ secondary amine synthesized as described above. This mixture was refluxed in ethanol for days to synthesize the tricyclic nucleobase (figure 2-0-17). The outcome of this reaction was the debenzylation of the starting material and no nucleophilic aromatic substitution product was recovered. To ensure that the product was not just going undetected, the reaction was scaled up, but this resulted again in only detectable debenzylation. In the absence of the cesium carbonate, the reaction conditions resulted in evidence of trace amounts of a new compound by NMR along with a large amount of unreacted starting material. This product was not recoverable following chromatography. Similar NMR evidence of possible product formation was seen if the reaction was performed in butanol to allow heating to 101 °C. Under these higher temperature conditions there was also evidence of starting material debenzylation. Using the originally reported fluorine source of potassium fluoride in ethanol did not result in any reaction of the tetC⁰ secondary amine starting material.

The small amounts of tricyclic nucleobase that were synthesized were subjected to deprotection conditions to remove the benzyl group so that the nucleobases could be glycosylated. Refluxing benzyl protected 8-Cl-tC⁰ nucleobase in either a 1:1 mixture of benzene and toluene or xylenes, with boron tribromide afforded small amounts of the free nucleobase (figure 2-0-24). The oxygen of the C2 carbonyl nucleophilically attacks the boron releasing one of the bromines as an anion. This anion attacks the benzyl CH2 carbon, displacing the N1 nitrogen. The electrons from this nitrogen resonate to form a double bond with the C2 carbon and alleviate the positive charge of the oxygen. Upon
workup, the boron is removed, reforming the carbon oxygen double bond and pushing the electrons back on to the nitrogen. N1 becomes protonated to give the free nucleobase product. Under the same conditions, the 8-F-tC° nucleobase retained its benzyl protecting group. To understand the different outcomes, the benzyl protected 7-Cl-tC° secondary amine was reacted under these conditions. This resulted in a small amount of debenzylated secondary amine product being recovered from the reaction (figure 2-0-24).

The lack of the lability of the benzyl protecting group was further investigated with some alternative debenzylation conditions. Standard conditions for the removal of the benzyl group by hydrogenation of the protected 7-Cl-tC° secondary amine with palladium on charcoal in methanol did not result in the debenzylation of this compound (figure 2-0-25). The palladium undergoes oxidative addition inserting itself in the nitrogen benzyl carbon bond. Hydrogen is coordinated by the palladium and the electrons of the bond between the nitrogen and the palladium take the hydronium while the hydride is transferred to the palladium. This releases the deprotected nucleobase and toluene is released during the reductive elimination to return the palladium to the catalytic cycle.

Using ammonium formate as the hydrogen source in debenzylation of this secondary amine did show evidence of reaction through the appearance of a new
compound by thin layer chromatography. Unfortunately, no product was isolated from purification.

Figure 2-0-25: Alternative debenzylation. Other synthetic conditions applied to the removal of benzyl protecting group from secondary amine intermediates. Reactions were unsuccessful.

Debenzylation was also attempted using aluminum trichloride in benzene as opposed to borontribromide. The protected 7-Cl-tC₀ secondary amine showed no reaction in this case and this may have been due to a lack of solubility of the starting material (figure 2-0-25).

Difficulties in debenzylation led to a re-examination of protecting group choice for 5-bromouracil. An Fmoc protecting group might have been a protecting group that would be labile enough to be removed following ring closure but not so reactive to the secondary amine formation conditions as some of the other esters.⁹⁴ For protection, n-methyl morpholidate deprotonates the N1 nitrogen so it can attack the carbonyl carbon of the Fmoc-Cl and upon reformation of the carbon oxygen double bond the chlorine anion is expelled (figure 2-0-26).⁹⁵ Although thin layer chromatography of the reaction showed a complex mixture of products, following multiple rounds of purification it was only 5-bromouracil with free N1 nitrogen that was recovered.
Figure 2-0-26: Fmoc protection of 5-bromouracil. Synthetic conditions presented for the installation of an alternative protecting group at N1 nitrogen of 5-bromouracil. This group would be easier to remove following secondary amine formation. Reaction was unsuccessful.
Oxygen Tricyclic Cytosine Analogs Experimental:

1-tert-butoxycarbonyl-5-bromouracil. 5-bromouracil (1.53 g, 8.0 mmol) was dissolved in acetonitrile (50 mL) under N₂. Dimethylaminopyridine (10.3 mg, 0.084 mmol) was added followed by di-tert-butyl dicarbonate (1.73 g, 7.95 mmol) and stirred at room temperature until no starting material was detectable by thin layer chromatography. Evaporation and purification via flash silica gel chromatography (CH₃OH in CH₂Cl₂) resulted in desired product (1.17 g, 4.0 mmol) 50% yield. ¹H NMR (CD₃OD) δ 8.28 (s, 1H), 1.60 (s, 9H).

1-ethoxycarbonyl-5-bromouracil. 5-bromouracil (191 mg, 1 mmol) was dissolved in dichloromethane (25 mL) under N₂. Ethyl chloroformate (95 μL, 1 mmol) was added followed by potassium carbonate (226 mg, 1.63 mmol) and this was stirred at room temperature for 14 hours. An hour of heating to 40 degrees resulted in the reaction of all of the starting material, and the mixture was quenched with saturated ammonium chloride (10 mL). After attempted extraction with ethyl acetate and dichloromethane aqueous and organics were evaporated. Purification via flash silica gel chromatography (Ethyl acetate in Cyclohexanes) resulted in desired product (82.6 mg, 0.31 mmol) 31% yield. ¹H NMR ((CD₃)₂SO) δ 11.52 (s, 0H), 7.90 (s, 1H).
**1-benzyl-5-bromoracil.** 5-bromouracil (15 g, 78 mmol) was dissolved in dichloroethane (75 mL) under N₂. Hexamethyldisilazane (22 mL, 104 mmol) was added followed by chloro-trimethylsilane (990 μL, 7.8 mmol) and heated to 85 °C. After 9.5 hours the solvent was removed. At room temperature the reaction was dissolved in dichloroethane (75 mL), iodine (208 mg, 819 mmol) was added followed by benzyl bromide (11 mL, 93.3 mmol) and the reaction returned to 85 °C. After 18 hours the reaction was quenched with water (120 mL). The desired product was filtered, washed with water and dichloromethane to give (16.3 g, 58 mmol) 74 % yield. 

\[
\begin{align*}
\text{1-HNMR } & & ((\text{CD}_3)_2\text{SO}) & \delta 8.35 \text{ (s, 1H), 7.31 (dt, } J = 7.7, 1.3 \text{ Hz, 5H), 4.87 (s, 2H).}
\end{align*}
\]

**1-benzyl-5-bromo-N4-(2-hydroxyphenyl)-cytosine.** Triphenylphosphine (93 mg, 0.36 mmol) was dissolved in dry dichloromethane (2 mL) with carbon tetrachloride (2 mL) under N₂. After 15 minutes at room temperature 1-benzyl-5-bromouracil (50 mg, 0.18 mmol) was added and the reaction heated to 44 °C. The reaction was cooled to room temperature after 5 hours and then to 0 °C. Aminophenol (20 mg, 0.18 mmol) with 1,8-Diazabicyclo[5.4.0]undec-7-ene (27 μL, 0.18 mmol) in dry dichloromethane (0.5 mL) was added to the reaction and stirred at 0 °C until starting material was no longer
detectable by thin layer chromatography. Dilution with methanol, evaporation and purification via silica gel flash chromatography followed by recrystallization from ethanol gave desired product (21 mg, 0.056 mmol) 32% yield. $^1$H NMR (CD$_3$OD) δ 8.43 (dd, J=8.1, 1.6 Hz, 1H), 8.14 (s, 1H), 7.69 – 7.60 (m, 1H), 7.60 – 7.52 (m, 1H), 7.42 – 7.27 (m, 5H), 6.99 (ddd, J=8.1, 7.3, 1.6 Hz, 1H), 6.91 – 6.82 (m, 2H), 5.03 (s, 2H).

$^{1,3}$-diaza-$^{1}$-benzyl-$^{2}$-oxophenoxazine. 1-benzyl-5-bromo-N4-(2-hydroxyphenyl)cytosine (15 mg, 0.040 mmol) was combined with potassium fluoride (23 mg, 0.40 mmol) under N$_2$ and dissolved in ethanol (1.5 mL). After refluxing 2 days, evaporation and purification via silica gel flash chromatography followed by recrystallization from ethanol gave product (0.4 mg, 0.0014 mmol) 3% yield. $^1$H NMR (CD$_3$OD) δ 7.40 – 7.26 (m, 5H), 6.90 – 6.83 (m, 2H), 6.77 (s, 2H), 4.93 (d, J=3.5 Hz, 2H).
**1-benzyl-5-bromo-N4-(2-hydroxy-5-fluorophenyl)-cytosine.** 1-benzyl-5-bromoracil (2.0 g, 7.1 mmol) was combined with 2-mesitylenesulfonyl chloride (1.5 g, 7.1 mmol) and dimethylaminopyridine (46 mg, 0.38 mmol) under N₂ and dissolved in dry dichloromethane (80 mL). Triethylamine (11 mL, 80 mmol) was added and stirred at room temperature 13 hours. 4-fluoro-2-aminophenol (1.2 g, 8.5 mmol) was combined with 1,8-Diazabicyclo[5.4.0]undec-7-ene (1 mL, 7.1 mmol) in dry dichloromethane to dissolve and added to the reaction above. Dilution with ethyl acetate, washing with saturated sodium bicarbonate, filtration and evaporation were followed by purification via silica gel flash chromatography (Ethyl acetate in Cyclohexanes) to give desired product (405mg, 1.0 mmol) 14% yield. \(^1\)HNMR (CD₃OD) δ 8.48 (dd, J=10.8, 3.1 Hz, 1H), 8.03 (s, 1H), 7.73 (s, 0H), 7.40 – 7.24 (m, 5H), 6.80 (dd, J=8.8, 5.1 Hz, 1H), 6.66 (ddd, J=8.8, 8.1, 3.1 Hz, 1H), 5.03 (s, 2H).
8-fluoro-1,3-diaza-1-benzyl-2-oxophenoxazine. 1-benzyl-5-bromo-N4-(2-hydroxy-5-fluorophenyl)cytosine (405 mg, 1.0 mmol) was combined with cesium fluoride (1.6 g, 10.4 mmol) under N$_2$ and dissolved in butanol (8 mL). After heating at 101 °C for 24 hours the reaction was evaporated and purification via flash silica gel chromatography (CH$_3$OH in CH$_2$Cl$_2$) gave crude product. $^1$HNMR ((CD$_3$)$_2$SO) δ 11.85 (s, 6H), 8.38 (s, 10H), 7.40 – 7.26 (m, 64H), 6.79 (s, 1H), 6.65(td, J=8.6, 3.0 Hz, 1H), 6.58 (dd, J=9.3, 3.0 Hz, 1H), 4.78 (s, 2H).

1-benzyl-5-bromo-N4-(2-hydroxy-5-methoxyphenyl)-cytosine. 1-benzyl-5-bromoracil (501 mg, 1.8 mmol) was combined with 2-mesitylenesulfonyl chloride (389 mg, 1.8 mmol) and dimethylaminopyridine (11 mg, 0.089 mmol) under N$_2$ and dissolved in dry dichloromethane (20 mL). Triethylamine (2.8 mL, 20 mmol) was added and stirred at room temperature 18 hours. 4-methoxy-2-aminophenol (300 mg, 2.2 mmol) was combined with 1,8-Diazabicyclo[5.4.0]undec-7-ene (265 μL, 1.8 mmol) in dry
dichloromethane to dissolve and added to the reaction above. Dilution with ethyl acetate, washing with saturated sodium bicarbonate, filtration and evaporation were followed by purification via silica gel flash chromatography (Ethyl acetate in Cyclohexanes) to give desired product (133mg, 0.33 mmol) 18% yield. $^1$HNMR ($\left(\text{CD}_3\right)_2\text{SO})$ δ 9.83 (s, 0H), 8.46 (s, 1H), 8.26 (s, 0H), 7.97 (s, 1H), 7.66 – 7.52 (m,1H), 7.39 – 7.26 (m, 5H), 6.82 (d, J=8.8 Hz, 1H), 6.58 (dd, J=8.8, 3.1 Hz, 1H), 4.94 (s, 2H), 3.67 (s,3H).

$8$-$\text{methoxy-1,3-diaza-1-benzyl-2-oxophenoxazine}$. 1-benzyl-5-bromo-N4-(2-hydroxy-5-methoxyphenyl)cytosine (133 mg, 0.33 mmol) was combined with cesium fluoride (502 mg, 3.3 mmol) under N$_2$ and dissolved in butanol (3 mL). After heating at 101 °C for 24 hours the reaction was evaporated and purification via flash silica gel chromatography (CH$_3$OH in CH$_2$Cl$_2$) gave crude product (1.3 mg, 0.0036 mmol).

$1$-$\text{benzyl-5-bromo-N4-(2-hydroxy-5-chlorophenyl)-cytosine}$. 1-benzyl-5-bromoracil (2.0 g, 7.1 mmol) was combined with 2-mesitylenesulfonyl chloride (1.5 g, 7.1 mmol) and dimethylaminopyridine (47 mg, 0.38 mmol) under N$_2$ and dissolved in dry dichloromethane (80 mL). Triethylamine (11 mL, 80 mmol) was added and stirred at
room temperature 18 hours. 4-Chloro-2-aminophenol (1.2 g, 8.5 mmol) was combined with 1,8-Diazabicyclo[5.4.0]undec-7-ene (1 mL, 7.1 mmol) in dry dichloromethane to dissolve and added to the reaction above. Dilution with ethyl acetate, washing with saturated sodium bicarbonate, filtration and evaporation were followed by purification via silica gel flash chromatography to give desired product (900mg, 2.1 mmol) 30% yield. \(^1\)HNMR ((CD\(_3\))\(_2\)SO) δ 10.68 (s, 1H), 8.50 (s, 1H), 8.37 (d, J=2.6 Hz, 1H), 8.26 (s, 1H), 7.40–7.29 (m, 5H), 7.03 (dd, J=8.6, 2.6 Hz, 1H), 6.92 (d, J=8.6 Hz, 1H), 4.95 (s, 2H).

8-chloro-1,3-diaza-1-benzyl-2-oxophenoxazine. 1-benzyl-5-bromo-N4-(2-hydroxy-5-chlorophenyl)cytosine (300 mg, 0.70 mmol) was combined with cesium fluoride (1.1 g, 7.0 mmol) under N\(_2\) and dissolved in ethanol (6 mL). After refluxing for three days the reaction was diluted with methanol and evaporated. Purification via flash silica gel chromatography (CH\(_3\)OH in CH\(_2\)Cl\(_2\)) gave product (38 mg, 0.12 mmol) 17% yield. \(^1\)HNMR ((CD\(_3\))\(_2\)SO) δ 7.39 – 7.25 (m, 6H), 6.86 (dd, J=8.6, 2.5 Hz, 1H), 6.81 – 6.74 (m, 2H), 4.78 (s, 2H).

85
86

**1-benzyl-5-bromo-N4-(2-hydroxy-4-chlorophenyl)-cytosine.** 1-benzyl-5-bromoracil (2.0 g, 7.1 mmol) was combined with 2-mesitylenesulfonyl chloride (1.5 g, 7.1 mmol) and dimethylaminopyridine (47 mg, 0.38 mmol) under N₂ and dissolved in dry dichloromethane (80 mL). Triethylamine (11 mL, 80 mmol) was added and stirred at room temperature 18 hours. 4-Chloro-2-aminophenol (1.2 g, 8.5 mmol) was combined with 1,8-Diazabicyclo[5.4.0]undec-7-ene (1 mL, 7.1 mmol) in dry dichloromethane to dissolve and added to the reaction above. Dilution with ethyl acetate, washing with saturated sodium bicarbonate, filtration and evaporation were followed by purification via silica gel flash chromatography to give desired product (1.5 g, 3.5 mmol) 49% yield. 

\(^1\)HNMR (\((CD_3)_{2}SO\) δ 11.86 (s, 1H), 9.48 (s, 0H), 8.38 (s, 1H), 7.40 – 7.30 (m, 5H), 6.63 (t, J=1.2 Hz, 1H), 6.55 (d, J=1.7 Hz, 2H), 4.88 (s, 2H).

**7-chloro-1,3-diaza-1-benzyl-2-oxophenoxazine.** 1-benzyl-5-bromo-N4-(2-hydroxy-4-chlorophenyl)cytosine (200 mg, 0.47 mmol) was combined with cesium fluoride (710 mg, 4.7 mmol) under N₂ and dissolved in ethanol (4 mL). After refluxing
for three days the reaction was diluted with methanol and evaporated. Purification via flash silica gel chromatography (CH$_3$OH in CH$_2$Cl$_2$) gave crude product.

\[
\text{1-benzyl-5-bromo-N4-(2-hydroxynaphthol)-cytosine.}
\]

1-benzyl-5-bromoracil (500 mg, 1.8 mmol) was combined with 2-mesitylenesulfonyl chloride (388 mg, 1.8 mmol) and dimethylaminopyridine (12 mg, 0.089 mmol) under N$_2$ and dissolved in dry dichloromethane (20 mL). Triethylamine (2.7 mL, 20 mmol) was added and stirred at room temperature 18 hours. Aminonaphthol (338 mg, 2.2 mmol) was combined with 1,8-Diazabicyclo[5.4.0]undec-7-ene (265 μL, 1.8 mmol) in dry dichloromethane to dissolve and added to the reaction above. Dilution with ethyl acetate, washing with saturated sodium bicarbonate, filtration and evaporation were followed by purification via silica gel flash chromatography (Ethyl acetate in Cyclohexanes) to give desired product (164mg, 0.39 mmol) 21% yield. $^{1}$HNMR (CD$_3$OD) δ 9.22 (s, 1H), 8.08 (s, 1H), 7.99 (s, 0H), 7.81 (ddd, J=7.9, 1.5, 0.7Hz, 1H), 7.78 (s, 1H), 7.57 (dd, J=8.1, 1.3 Hz, 1H), 7.41 – 7.34 (m, 5H), 7.32 – 7.25 (m, 2H), 7.17 (s, 1H), 5.06 (s, 2H).
**1,3-diaza-1-benzyl-benzo(h)2-oxophenoxazine.** 1-benzyl-5-bromo-N4-(2-hydroxynaphthol)-cytosine (164 mg, 0.39 mmol) was combined with cesium fluoride (591 mg, 3.9 mmol) under N\textsubscript{2} and dissolved in butanol (4 mL). After heating at 101 °C for 3 days the reaction was evaporated and purification via flash silica gel chromatography (CH\textsubscript{3}OH in CH\textsubscript{2}Cl\textsubscript{2}) gave crude product (0.8 mg, 0.002 mmol).

**8-chloro-1,3-diazal-2-oxophenoxazine.** 8-chloro-1,3-diaza-1-benzyl-2-oxophenoxazine (20 mg, 0.61 mmol) was dissolved in benzene (1.6 mL) and toluene (1.6 mL) under N\textsubscript{2}. 1M Boron tribromide (300 μL, 0.3 mmol) solution in dichloromethane was added to the reaction and heated to 110 °C for 4.25 hours. Evaporation and purification via flash silica gel chromatography resulted in product (6.7 mg, 0.28 mmol) 46% yield. \textsuperscript{1}HNMR (CD\textsubscript{3}OD) \(\delta\) 7.75, 7.63.
Carbon Tricyclic Cytosine Analogs

A carbon based tricyclic cytosine (tCtCt) is a target of interest because of its potential fluorescence properties and its ability to present a triple acceptor hydrogen bonding pattern. This bonding pattern would be potentially suitable for making new base pairs (figure 2-0-27). Its synthesis was claimed in the literature regarding testing for its analgesic abilities, but there is little known as to its fluorescence properties. Extensive testing of the reported routes in the laboratory has called into question the possibility that the claimed molecules were those that were tested. Some new attempts at this molecule will now be described.

Acetanilide was the initial starting material for this synthesis and it was combined with phosphorous oxychloride in DMF to form the two distal rings of the nucleobase (figure 2-0-28). In Vilsmeier chemistry, as seen in the synthesis of the oxygen analogs above, the phosphorous oxychloride is attacked by the oxygen of the DMF with the assistance of the amide nitrogen. This results in the leaving of one of the phosphorous oxychloride chlorines as the anion. Chlorine then substitutes at the amide carbon of DMF releasing the dichlorophosphate anion and generating the reactive iminium cation.
This iminium cation is attacked at carbon by the $\pi$ system of acetanilide and the following deprotonation restores aromaticity. The chlorine of the iminium then leaves, regenerating the cation. The enol tautomer of the acetanilide amide can attack the iminium carbon forming the second ring of the ensuing nucleobase and elimination releases diethylamine. The resulting double bond of this elimination can attack another iminium cation resulting from the reaction of DMF and POCl$_3$. The double bond is restored by deprotonation. The amide carbonyl reacts with phosphorous oxychloride in a manner similar to that of DMF, resulting in chlorine substitution and a carbon nitrogen
bond in place of the carbonyl. Aqueous workup results in the hydrolysis of the remaining iminium cation and the formation of the carbaldehyde of the aryl quinolone.

![Chemical structure](image)

*Figure 2-0-29: Quinolinamine synthesis. This is the second synthetic step in the synthesis of tC*.

The formylquinoline thus produced was then reacted with the hydrochloride salt of guanidine to give the quinolinamine which is the desired tricyclic structure. Imine bond formation between the guanidine nitrogen and the aldehyde of the formylquinoline, combined with nucleophilic aromatic substitution by the other guanidine nitrogen at the chlorine forms the product, increasing its nucleophilicity and it will attack the carbonyl carbon of the aldehyde.

Previous attempts in the lab to make the compound with exocyclic oxygen at the C2 position were unsuccessful, so this nitrogen version was synthesized to be later converted to the oxygen containing compound. During early synthesis attempts potassium carbonate was used to facilitate the nucleophilic attack of guanidine. Potassium Carbonate in triethylamine was also tried, followed by sodium hydride. This hydride was the base of choice because it could result in product when conditions were extremely dry.

![Chemical structure](image)

*Figure 2-0-30: Nitrosation. This is the third synthetic step in the synthesis of tC*. The quinolinamine is converted to the quinolinol which has the correct nucleobase hydrogen bonding face.

Attempted nitrosation of the quinolinamine to the quinolinol was made through the use of acid followed by sodium nitrite and then base (figure 2-0-30). This is an
established method for the synthesis of synthetic nucleosides, taking advantage of well precedentied diazonium chemistry.\textsuperscript{99} Dissociation of the nitrite allows the nitrite oxygen anion to pick up protons from the acidic environment. Upon the second protonation, water is formed and the group departs leaving nitric oxide. The primary amine of the quinolinamine attacks the positively charged nitrogen of the nitric oxide and is then deprotonated. Proton transfer of the other amine hydrogen to the oxygen of the nitric oxide followed by additional protonation leads to water. Leaving of this water, generates the diazonium intermediate which is highly reactive to substitution by the hydroxide resulting in the alcohol product. Acetic acid performed better than HCl to give the desired product in these conditions.\textsuperscript{100} Additionally, greater reactivity was observed when the sodium nitrate was added as an aqueous solution, likely due to the increased solubility. This reaction was performed in a pressure tube, but the sealed environment was not shown to increase reaction performance.

Possible decomposition of the amine starting material during storage was avoided through trapping the compound with an amine protecting group (figure 2-0-31). Boc protection should be labile to the nitrosation conditions and so this protecting group was installed on the quinolinamine. Starting material was combined with boc anhydride in dry THF to yield the protected product through the mechanism discussed earlier for 5-bromouracil protection. Reaction of this protected amine in acetic acid with aqueous sodium nitrate led to the desired product as confirmed by mass spectroscopy.
Figure 2-0-31: Boc protected nitrosation. These conditions were used to stabilize the quinolinamine to avoid decomposition prior to conversion to the quinolinol which has the correct nucleobase hydrogen bonding face.
Carbon Tricyclic Cytosine Analogs Experimental:

2-chloro-3-formylquinoline. Dimethylformamide (2.8 mL, 0.037 mol) was cooled to 0 °C under N₂. Phosphorous oxychloride (9.6 mL, 0.10 mol) was added. After warming to room temperature, acetanilide (2 g, 0.015 mol) was added and the reaction heated to 75 °C. 4 hours later the reaction mixture was added dropwise to ice and stirred. Precipitated product was filtered (613 mg, 0.0032 mmol) 21% yield.

Pyrimido[4,5-b]quinolin-2-amine. Guanidine hydrochloride (1.8 g, 19 mmol) was dissolved in DMF (22 mL) over 3 or 4 Å molecular sieves under N₂. This solution (15 mL) was transferred to a new flask over new sieves and sodium hydride (124 mg, 12 mmol) was added to the reaction and stirred for 30 minutes. 2-chloro-3-formylquinoline (600 mg, 3.1 mmol) was added and the reaction heated to 80 °C. After 3 hours the reaction was cooled to room temperature, filtered through celite, washed with methanol and evaporated. Purification via silica gel chromatography yielded product (419 mg, 2.1 mmol) in 68% yield.
Pyrimido[4,5-b]quinolin-2-tert-butoxycarbonylamine. Pyrimido[4,5-b]quinolin-2-amine (146mg, 0.74 mmol) was dissolved in THF (33 mL) under N₂. Di-tertbutyl bicarbonate (663 mg, 3.0 mmol) was added to the reaction and heated to 40 °C. After additional di-tertbutyl bicarbonate (1.3 g, 5.8 mmol) was added and 20 hours, the reaction was evaporated. Purification via silica gel chromatography resulted in the product (12.6 mg, 0.042 mmol) 6% yield.

Pyrimido[4,5-b]quinolin-2-ol. Pyrimido[4,5-b]quinolin-2-tert-butoxycarbonylamine (6 mg, 0.020 mmol) was dissolved in 10% acetic acid (1 mL) and stirred for 20 minutes. Sodium nitrite (3.4 mg, 0.051 mmol) was added to the reaction and stirred at room temperature for 90 minutes. More sodium nitrite (4.0 mg, 0.058 mmol) was added to the reaction and stirred another 90 minutes. Heating to 60 °C the reaction was continued for 90 minutes and then neutralized with triethylamine (0.243 mL, 0.0017 mmol) Purification via preparatory thin layer chromatography resulted in the product.
Chapter Three: Nucleoside Directed Synthesis

Introduction

In this chapter will be a discussion of synthetic approaches to tricyclic cytidines using nucleosides as starting materials (figure 3-0-1). The majority of the chapter focuses on the oxygen substituted analogs (tC\textsuperscript{9} derivatives), but elaborations of both the oxygen and sulfur containing compounds (tC\textsuperscript{9} and tC derivatives) are discussed as well as some chemistry of other fluorescent nucleotides. Through these synthetic methods, it was ultimately possible to obtain a set of pure nucleoside analogs which were subsequently tested for photophysical properties as will be discussed in Chapter 4. Although substituent effects had great impacts on the chemistry and many reaction conditions tested had limited substituent tolerance, the methods developed were the most reliable way to synthesize satisfactory yields of the entire small library of compounds. Some reactions were developed with uniquely high yields for a single nucleoside analog, and others were found to have usefully broad substrate scope.
The presence of the somewhat labile glycosidic bond made the nucleoside directed synthesis less tolerant to harsh reaction conditions. A glycosylated starting material endeavor was the known synthetic route to the unsubstituted oxygen tricyclic cytosine analog tC\(^{\alpha}\) and therefore a reasonable avenue to the new substituted versions.\(^3\) Synthetic routes in which these compounds should be stable occasionally resulted in the fracture of this bond, but eventually this sensitivity was managed, and the glycosidic bond could be retained throughout the synthesis. Additionally, deamination like that seen under reaction conditions in the previous chapter was also a problem with the synthesis of nucleosides.
Obtaining the nucleosides was not the end of the synthetic work, as attempts to incorporate the fluorophores into nucleic acids required additional synthetic manipulation (figure 3-0-2). Other synthetic manipulations of the nucleosides were tested to further modify the character of the chromophore and alter the photophysics after the completion of the tricyclic system. A series of triphosphates of the nucleoside analogs were synthesized in preparation for enzymatic incorporation into oligonucleotides. Triphosphates of these analogs provide means to investigate enzyme utility (fidelity and rates of nucleotide incorporation of these novel nucleotides as well, as will be discussed in Chapter 5 of this work.
Oxygen Tricyclic Cytidine Analogs

5-bromo-2’-deoxy-uridine is the starting material for the previously published synthetic route to tC⁰, and many low yielding reactions in the course of optimization motivated the synthesis of this commercially available starting material. Bromination was achieved in good yields even when on large scale with dibromo-dimethylhydantoin (figure 3-0-1). This brominating reagent behaves similarly to N-bromosuccinimide, but is less expensive and less hazardous. The N1 nitrogen of bromouridine resonates to form a double bond with the C6 carbon and the C5-C6 double bond on the nucleoside attacks the electrophilic bromine of the DBH. The nitrogen anion thus formed deprotonates the C5 carbon to reform the C5-C6 double bond and restore the N1 nitrogen lone pair.

It is unnecessary to protect the 3’ and the 5’ hydroxyl groups from these bromination conditions, but leaving free this functionality hindered reaction with the carbonyl activators in secondary amine formation. To prevent this undesirable interference, these oxygens were tied up in protecting groups as the next synthetic step. Acetyl protecting groups were useful for the protection at these positions because they are base labile. Acetic anhydride in pyridine provided the double protected product in good yields. The reaction could be catalyzed with a small amount of DMAP, like the boc protection conditions in the previous chapter, but this was not necessary. In the presence of DMAP, the carbohydrate oxygen substitutes for DMAP at the carbonyl carbon of what was the anhydride. DMAP nucleophilic attack of the acetic anhydride and reformation of the carbonyl double bond released an acetate anion which could deprotonate the carbohydrate oxygen. The 3’ OH underwent the same process. In the absence of DMAP,
the hydroxyls attack directly the carbonyl carbon of acetic anhydride. In the absence of these protecting groups, low yields result even from the most successful activation conditions as will be discussed below.

A comparative standard was helpful during the synthesis of the tC⁰ secondary amines, to determine if the desired products were being formed. To have on hand the intermediates from the synthesis of the parent compound was a helpful resource. The synthesis of this compound is found in the literature and is discussed in the previous chapter for its application towards the synthesis of the nucleobases.³ A 25mg scale synthesis proved too small to provide thin layer chromatography standards of synthetic intermediates, but use of a 200mg scale afforded the desired model compounds. To determine if the synthesis of this parent compound could be improved through some alternative carbonyl activation conditions a small investigation was made.

Using the Appel chemistry discussed in the previous chapter, 3’,5’-diacetyl-5-bromo-2’-deoxyuridine was added to a mixture of triphenyl phosphine and carbon tetrachloride (figure 3-0-4). After addition of the aminophenol and DBU, no nucleophilic substitution was observed. Phosphorous oxychloride conditions, also discussed in the previous chapter, were applied to synthesis of the unsubstituted tC⁰ nucleoside (figure 3-0-5). The protected nucleoside starting material was reacted with a mixture of phosphorous oxychloride and n-methylimidazole, leading to the appearance of product by thin layer chromatography. The yield of the isolated desired product from this reaction was not an improvement on the established sulphonylating conditions, which are selective for C4 due to steric.
For fluorescence measurement comparison it was necessary again to synthesize unsubstituted tC° using the literature method. The yields were greatest when the reaction time between the sulphonylated-bromouridine intermediate and aminophenol was limited to two hours. Isolation from unreacted starting materials is aided by the removal of the acyl protecting groups. Additionally, the presence of free alcohol at the 5’ position is useful in the following synthetic step so the semi-pure reaction mixture was deprotected through the literature method.³ Reaction with sodium methoxide in methanol efficiently cleaves the ester protecting groups from both the 3’ and 5’ positions while generally leaving the other functionality of the secondary amine intact. Methoxide anions attacked the carbonyl carbons of the protecting groups and reformation of the double bonds expelled methyl esters to free 3’ and 5’ carbohydrate oxygens.¹⁰⁶ Acidic workup of the reaction led to the protonation of the oxygens to give the hydroxyl product. The following is a table (table 3) of secondary amine formation conditions tested for the oxygen tricyclic cytosine (tC°) analogs.
Table 3: Nucleoside secondary amine formation. Analog is the name of the substituted product referring to figure 3-0. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
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<th>Analog</th>
<th>Activator</th>
<th>Solvent</th>
<th>Temperature</th>
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<th>Substitution Time</th>
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Table 3 Continued: Nucleoside secondary amine formation. Analog is the name of the substituted product referring to figure 3-0-3. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
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<th>Analog</th>
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</thead>
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<td>7-Ome</td>
<td>PPh3</td>
<td>DCM/CCL4</td>
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<td>9 hrs</td>
<td>20min at 0</td>
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<td>14</td>
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</tr>
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<td>DCM/CCL4</td>
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<td></td>
<td></td>
<td>500mg</td>
<td>23</td>
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</tr>
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<td>MesCl</td>
<td>DCM</td>
<td>RT</td>
<td>overnight</td>
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<td>NA</td>
<td>NA</td>
</tr>
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<td>MesCl</td>
<td>DCM</td>
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<td>overnight</td>
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<td>NA</td>
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<tr>
<td>8-Cl</td>
<td>PPh3</td>
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<td></td>
<td>15min at 44</td>
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</tr>
<tr>
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<td>rt</td>
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</tr>
<tr>
<td>8-Cl</td>
<td>PPh3</td>
<td>DCM/CCL4</td>
<td>44</td>
<td></td>
<td>5 hrs</td>
<td>1 at 0 the 44</td>
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<td>mono</td>
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<td>PPh3</td>
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<td>44</td>
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<td>1 hr at 44</td>
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<td>44 for 15min</td>
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<td>DCM/CCL4</td>
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<td>4.5hrs</td>
<td>at 44</td>
<td>500mg</td>
<td>38</td>
</tr>
<tr>
<td>8-Cl</td>
<td>PPh3</td>
<td>DCM/CCL4</td>
<td>44</td>
<td></td>
<td>15min at 44</td>
<td>500mg</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td>8-F</td>
<td>MesCl</td>
<td>DCM</td>
<td>RT</td>
<td>overnight</td>
<td>4hrs</td>
<td>25mg</td>
<td>0</td>
<td>NA</td>
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<td>MesCl</td>
<td>DCM</td>
<td>RT</td>
<td>overnight</td>
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<td>150mg</td>
<td></td>
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</tr>
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<td>DCM</td>
<td>RT</td>
<td>overnight</td>
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<td>250mg</td>
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<td>overnight</td>
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<td>8-F</td>
<td>MesCl</td>
<td>DCM</td>
<td>RT</td>
<td>overnight</td>
<td>overnight</td>
<td>500mg</td>
<td>NA</td>
<td></td>
</tr>
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<td>DCM/CCL4</td>
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<td>3hrs</td>
<td>overnight rt</td>
<td>1g</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>8-F</td>
<td>PPh3</td>
<td>DCM/CCL4</td>
<td>44</td>
<td>5 hrs</td>
<td>1hr at 0</td>
<td>500mg</td>
<td>38</td>
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</tbody>
</table>
Table 3 Continued: Nucleoside secondary amine formation. Analog is the name of the substituted product referring to figure 3-0-3. Some yields are approximations based on NMR and in the event that no yield is given, isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Activator</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Activation</th>
<th>Substitution</th>
<th>Scale</th>
<th>Yield</th>
<th>MS</th>
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</tr>
<tr>
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<td>DCM/CCL4</td>
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<tr>
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<td>0 then rt</td>
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<td>24</td>
<td></td>
</tr>
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<td>DCM/CCL4</td>
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<td>6 hrs</td>
<td></td>
<td>500mg</td>
<td>NA</td>
<td></td>
</tr>
<tr>
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<td>DCM/CCL4</td>
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<tr>
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<td>DCM/CCL4</td>
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<td>500mg</td>
<td>NA</td>
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<td>DCM/CCL4</td>
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<td>8 hrs</td>
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<td>25mg</td>
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<td>overnight</td>
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<td>rt</td>
<td>850mg</td>
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Table 3 Continued: Nucleoside secondary amine formation. Analog is the name of the substituted product referring to figure 3-0-3. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
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<th>Solvent</th>
<th>Substitution</th>
<th>Scale</th>
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<td>NA</td>
<td>NA</td>
<td>4hrs</td>
<td>40</td>
<td>DCM/DOM/CCl4</td>
<td>overnight</td>
<td>1g</td>
</tr>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
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<td>500mg</td>
<td>DCM/DOM/CCl4</td>
<td>overnight</td>
<td>4</td>
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<td>16</td>
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<td>0.4-0.5</td>
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<td>overnight</td>
<td>13</td>
</tr>
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<td>15min at 0</td>
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<td>NA</td>
<td>5hrs</td>
<td>0.4-0.5</td>
<td>DCM/DOM/CCl4</td>
<td>overnight</td>
<td>13</td>
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*Note: Some scales were not provided in the original data.*
Table 3. Nucleoside secondary amine formation. Analog is the name of the substituted product referring to figure 3-30. Some yields are approximations based on NMR and in the event that no yield is given, isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

<table>
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<th>Activation</th>
<th>Substitution</th>
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<td>DCM/CCL4</td>
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<td>15min at 0</td>
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<tr>
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<td>PPh3</td>
<td>DCM/CCL4</td>
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<td>500mg</td>
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<td>DCM/CCL4</td>
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<td>5 hrs</td>
<td>15min at 0</td>
<td>500mg</td>
<td>44</td>
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</tr>
<tr>
<td>Tet</td>
<td>PPh3</td>
<td>DCM/CCL4</td>
<td>44</td>
<td>4.5 hrs</td>
<td>15min at 0</td>
<td>500mg</td>
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<td>DCM/CCL4</td>
<td>44</td>
<td>14min at 0</td>
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</tr>
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</tr>
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<td></td>
<td></td>
<td></td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>80 ovrnt</td>
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Initial attempts to make the tetracyclic secondary amine through the use of the established tC\textsuperscript{\textcircled{o}} procedure with aminonaphthol did not result in the formation of the desired product. Use of 2-mesitylenesulfonyl chloride to activate the carbonyl, followed by substitution with aminonaphthol in the presence of DBU, resulted initially in the appearance of potential secondary amine as shown by thin layer chromatography. Evidence of a promising new compound was also present in proton NMR of the crude reaction mixture. However, following purification aminonaphthol was the major compound recovered. Repeating the reaction under the same conditions but modifying the mobile phase of purification resulted in the recovery of a mixture that resembled that of the crude reaction, so deamination as a result of purification had been avoided. Mass spectroscopy of this crude mixture could not confirm the presence of the desired secondary amine. The mixture was subjected to the standard deprotection conditions discussed above (sodium methoxide in methanol), to attempt to make isolation easier. The product from this reaction was utilized as a TLC standard for scale up of the reaction. On a larger scale the same crude mixture was produced, but following the deprotection conditions the apparent product remained prohibitively difficult to isolate.
Decreasing the reaction time between the sulfonylmesitylene-bromouridine intermediate and the aminonaphthol, as was shown to improve yield with the parent compound, did not result in an increase in the recovered tetC\textsuperscript{0} secondary amine. Utilizing purification by preparatory thin layer chromatography it was possible to confirm the presence of the desired product by mass spectroscopy, even though it remained contaminated by the starting materials. Subjecting a larger scale reaction to these purification conditions and then removing the protecting groups resulted in the recovery of a larger amount of the desired compound, but it remained contaminated with 5-bromo-2’-deoxyuridine.

Due to these isolation and low yield issues, an alternative method for activation of the C4 carbonyl to substitution by the amine of aminonaphthol was investigated. Using the Appel conditions it was possible to recover the desired product as a mixture with triphenyl phosphine oxide. Deprotection of this product followed by purification lead to the removal of triphenyl phosphine oxide, but the desired product was contaminated with 5-bromo-2’-deoxyuridine. Increasing the scale of the reaction in an effort to have as much product to carry further as possible, resulted in a counterproductive decrease in yield of the desired product. Returning to the original scale and altering the
chromatographic mobile phase from ethyl acetate/methanol to cyclohexanes/ethyl acetate resulted in some product as well as some potentially partially deprotected product. Following subjection of the entire mixture to deprotection, a modest yield of isolated tetC\textsuperscript{o} secondary amine was obtained.

Believing that the conditions had been reasonably improved, scale up was again attempted considering cyclohexanes/ethyl acetate chromatographic mobile phase. Following flash chromatography, the recovered mixture which contained the desired product was triturated with ethanol. This trituration appeared to improve the purity of the compound, but when the product of trituration was subjected to the deprotection conditions deamination ensued. Purified, 5-bromo-2’-deoxyuridine was all that was recovered from chromatography of the deprotection reaction mixture. Reducing the scale slightly led to the same results following deprotection, even though the trituration was avoided. The original 500 mg scale was therefore revisited. Decreasing the reaction time and temperature between the 5-bromo-4-chloro-2’deoxy-uridine intermediate and the aminonaphthol resulted in an increase in the product recovered following deprotection. Changing the reaction time at this lower temperature had adverse effects on the production of desired product.

The ideal reaction time for this second step of the synthesis was determined to be 15 minutes. Altering the time of activation of the 5-bromo-2’-deoxyuridine with the triphenyl phosphine also showed some influence on the recoverable amount of the product, with the ideal reaction time at 44 degrees appearing to be 5 hours. Chromatographic conditions were optimized with a selected mobile phase of
dichloromethane and methanol that produces a mixture of desired product with triphenyl phosphine oxide. This mixture is resolvable chromatographically following deacetylation. Following deprotection, multiple purifications can be necessary to remove 5-bromo-2’-deoxy-uradine which is either the result of: some deamination during the deprotection conditions, some unreacted 3’,5’-diacetyl-5-bromo-2’-deoxyuridine which was carried through from the previous synthetic step and then deprotected, or a combination of both of these.

Although the improvement of yield was significant for these conditions over those for the synthesis of the parent compound some alternatives were also tried. BOP and PYBOP which are commonly used for peptide coupling were tested for utility as activators for substitution. These do not react with amines, which could cut down on possible fruitless side reactions. Although proton NMR showed some evidence of product formation there was no product recovered following deprotection in either of these experiments. Following the BOP reaction it was possible to recover the unreacted starting materials indicating that little reaction was taking place or deamination was rampant.

To synthesize the secondary amine of 7-Cl-tC°, 5-chloro-2-aminophenol was substituted for aminophenol in the conditions for the synthesis of tC° (figure 3-0-3). Activation with the 2-mesitylenesulfonyl chloride followed by addition of the chloroaminophenol resulted in a mixture of many products. This mixture was purified by flash silica gel chromatography followed by preparative thin layer chromatography. A mixture of one potential product and 3’,5’-diacetyl-5-bromo-2’-deoxyuridine remained.
Sending this mixture for mass spectroscopy resulted in detection of the molecular ion for the desired secondary amine product. Extending the reaction times for activation and substitution with these conditions resulted in a more complex mixture that contained a similar amount of the product.

![Figure 3-0-5: Phosphorous oxychloride nucleoside synthesis. Alternative conditions for the synthesis of substituted secondary amines that were successful for synthesis with chloro-aminophenols.](image)

Attempting the phosphorous oxychloride conditions previously discussed resulted in a product that appeared different by proton NMR than that isolated under the parent compound conditions, but had the same mass as confirmed by mass spectroscopy (figure 3-0-5). Under these conditions, the product was produced in a similarly small yield as that recovered from the other conditions above. Scaling up this reaction, it was possible to isolate 40mg of product and recover a large amount of product that was a mixture with 3’,5’-diacetyl-5-bromo-2’-deoxyuridine. Deprotection of this mixture resulted in desired product in high yield.

The Appel chemistry was also attempted on the 500 mg scale which was useful in the synthesis of tetC⁰ secondary amine. These conditions resulted in the apparent product by proton NMR, but mass spectral analysis of the compound showed high molecular weight adducts that lacked the expected bromine isotope peaks. At a later date, the reaction was retried with activation at a higher temperature for a longer period of time,
followed by substitution at a lower temperature for a shorter stint. This resulted in a
mixture of triphenyl phosphine oxide and apparent product that was subjected to
deprotection conditions. Following this cleavage reaction it was possible to recrystallize
the desired product from ethanol. This ethanol recrystallization was applied to the
protected secondary amine following chromatography and appeared to clean up the
product slightly. It is possible to create a large amount of secondary amine through the
use of a multiple batch system where no reaction has more than 500mg of starting
material. These batches can be combined for purification. With the batch system and the
optimized chromatography conditions, discussed for other analogs above, it is possible to
recover mixtures of triphenyl phosphine oxide and product. These compounds can be
separated following the removal of the acyl groups from the 3’ and 5’ positions of the
secondary amine. Whether in a two batch or a four batch system the yields are
comparable. Decreasing the reaction time of the 5-bromo-4-chloro-2’deoxy-uridine
intermediate and chloro-aminophenol from fifteen minutes to ten minutes increased the
recovery of the desired product. Decreasing the reaction time for the activation of the
carbonyl did not result in the same positive effect, and the previously selected 5 hour
reaction time was maintained.

Synthesis of the secondary amine sister analog where the chlorine substituent is
located in the neighboring position was a more challenging endeavor. 8-Cl-tC⁰ was
attempted using the tC⁰ parent compound synthetic route as a starting point (figures 3-0-6, 3-0-3). A small scale test reaction resulted in a crude mixture that did not appear to
contain any of the secondary amine. On a larger scale it was possible to see the
appearance of potential product by thin layer chromatography in the course of the reaction and in the crude material by proton NMR. Unfortunately, following chromatography is was not possible to recover the product that had appeared.

Switching to the Appel activation method at 44 degrees followed by substitution at 0 degrees did not result in the appearance of any secondary amine product by thin layer chromatography (figure 3-0-4). The temperature was maintained at 44 degrees for the substitution and this resulted in the recovery of a mixture of product and 3’, 5’-diacetyl-5-bromouridine. Following multiple attempts at purification, the mixture remained with mostly unchanged composition. This mixture was subjected to the standard deacetylation conditions, resulting in the removal of only one of the ester groups, necessitating a repetition of deprotection. With substitution taking place at the same temperature, an attempt was made to hurry carbonyl activation. This decrease in first step reaction time did not result in an increase in the product recovered, but upon the change to the optimized chromatography conditions discussed above, secondary amine recovery was increased. Decreases in the substitution reaction time from one day to one hour and then from one hour to fifteen minutes resulted in corresponding increases in product yield.

Mass spectroscopy confirmation of the product was made from a four batch reaction in which activation was 5 hours and substitution 15 minutes at 44 degrees after adding chloroaminophenol mixture at 0 degrees.

A substituent in the same position with similar character was different enough to change the overall reactivity under these conditions. 8-F-tC° was the first new analog synthesized because it was the easiest to access through the parent analog synthesis
conditions (figure 3-0-6). A small scale test of the synthesis of the secondary amine resulted in the recovery of a complex mixture even following purification. Still, this mixture gave evidence that it may have contained some of the product. Scaling up under these conditions led to the isolation of some apparent product following purification. After subjecting this product to the deprotection conditions, any product had been deaminated and 5-bromo-2’-deoxyuridine was all that remained. Further increasing the scale of the reactants and shortening the time of substitution between the sulfonylated-bromouridine intermediate and the aminophenol resulted in product that could be isolated following deprotection. Mass spectroscopy confirmed that this isolated compound was indeed the desired product. Continued increase in scale resulted in apparently increasing yield of the secondary amine, but either the product was impure or was being deaminated during the deprotection conditions. Yields of the 3’ and 5’ hydroxyl secondary amine product were unusually low considering the apparent protected product that was being deprotected.

![Figure 3-0-6: Sulfination synthesis of nucleoside analogs. Literature synthetic method for formation of secondary amines that was useful in synthesis with flour-aminophenol.](image)

Initially the Appel conditions seemed to be a less effective route to the synthesis of 8-F-tC∞ secondary amine, as there was not a great deal of product produced and it was present as a mixture with a large amount of triphenylphosphine oxide (figure 3-0-4).
After extensive testing of conditions it was determined that the Appel chemistry could be higher yielding. Activation for five hours at 44 degrees and substitution at 0 degrees for 15 minutes gave a mixture of product and triphenylphosphine oxide. This mixture did not appear as sensitive to deamination during purification and/or under deprotection conditions. Substitution for a shorter amount of time resulted in a decrease in the yield of the desired product.

The related analog which has a methoxy functional group in the place of the fluorine had mass spectral confirmation of the desired secondary amine intermediate upon the first synthetic attempt. Reactions were made in parallel to compare the synthesis of 8-OMe-tC° under the Appel conditions and by the parent compound synthetic method (figure 3-0-6, 3-0-4). Both of these reactions resulted in the desired product, with the greater yield coming from the triphenyl phosphine reaction and the product of greater purity being isolable from the sulfonylating procedure. The product from the former was a mixture with a minor amount of triphenyl phosphine oxide and a large amount of unreacted methoxy-aminophenol. Deprotection of this mixture followed by purification gave the compound in fair yield. Scale up of this reaction, as was seen with the other analogs under these conditions, resulted in a significant decrease in yield. Using a batch method instead (a set of smaller scale reactions run in parallel), it was possible to synthesize hundreds of milligrams of secondary amine intermediate that could be isolated following deacetylation. To ensure that the maximum performance of these conditions was being observed, the reaction time for carbonyl activation was increased and the time
for substitution was decreased. These measures resulted in a decrease in the yield of the desired product, and so were discarded.

In one instance under these conditions, the protecting groups were lost from the carbohydrate moiety and this resulted in a significant decrease in the yield of the desired product. The protecting groups are likely preventing unwanted side reactions in both the activation and the substitution steps of secondary amine synthesis. With substitution at lower temperature the reaction was again tested for performance on a larger scale but this was unsuccessful. Combining the batch method with low temperature substitution it was possible to obtain pure product following deprotection in good yield.

As a check, the phosphorous oxychloride conditions were tried with the methoxy-aminophenol, although a large number of new compounds appeared to be produced during the reaction, there was no desired product isolated following purification. Another interesting procedure that was tried with this analog was the activation of the carbonyl for substitution using NCS and DMS. “Active DMSO” like that seen in Corey-Kim oxidation is formed when the sulfur of DMS abstracts the chlorine from NCS (figure 3-0-7). The nitrogen anion substitutes for the chlorine at sulfur, and this sulfur cation is activated to attack by the C4 carbonyl oxygen to release

![Figure 3-0-7: Active DMSO for secondary amine formation. Alternative conditions for the synthesis for substituted secondary amines that were not successful in activating 5-bromouridine to substitution.](image)
the succinimide. The succinimide can find a hydrogen from the N3 nitrogen. The aminophenol is then added to substitute at C4 and release DMSO. There was no substitution observed under these conditions.

When the methoxy substituent is located in the neighboring position to this analog (synthesis of 7-OMe-tC\textsuperscript{o}), so that the functionality is electron donating by resonance through its position \textit{para} to the amine of the aminophenol, it is a brilliant purple color and significantly more expensive. Due to the expense of this starting material, and its more limited commercially availability, its synthesis required less procedural optimization. The majority of the challenges had already been solved the synthesis of related compounds. Smaller scales were used for the synthesis of this analog, as a result of the aforementioned starting material availability. The first attempted synthesis of 7-OMe-tC\textsuperscript{o} was under the Appel conditions on a fairly small scale. The desired secondary amine product appeared to be isolated in a semi-crude state following chromatography of the reaction mixture. Attempting to repurify this semi-pure mixture resulted in recovery of a small amount of product the identity of which could not be confirmed. A larger scale reaction using the established multiple batch system (as used for other substituted tC\textsuperscript{o} analogs) was successful in producing a mixture of the product with 3’,5’-diacetyl-5-bromo-2’-deoxy-uridine and triphenylphosphine oxide. Following removal of the acetyl groups and multiple purifications it was possible to remove the triphenylphosphine oxide from this mixture. Further optimization resulted in a slight decrease in the time allowed for substitution of the methoxyaminophenol at the 3’5’diacetyl-5-bromo-4-chloro-2’deoxy-uridine intermediate. The multiple batch system was applied with these
optimized conditions to accumulate enough of this intermediate to proceed to the closure of the center ring and establishment of the chromophore. The mass of the product was confirmed following deprotection of the mixture from a two-batch reaction. One interesting approach that was attempted with this aminophenol was the attempted use of triphenyl phosphine oxide in the place of the triphenyl phosphine under the Appel conditions. Although there was evidence of a small amount of substitution, deprotection of this product resulted in deamination and deglycosylation.

Not unlike the synthesis of the nucleobase analogs of tC, a triazole intermediate of the nucleoside was targeted for synthesis, rationalized by its likely high reactivity to substitution (figure 3-0-8). After adding the phosphorous oxychloride and triethylamine to the reaction of triazole and 3',5'-diacetyl-5-bromo-4-chloro-2'-deoxyuridine and warming from 0 °C to room temperature there was no reaction. Heating the reaction to 40, 60 or even 80 °C could not cause reaction of the starting material. Changing the solvent from acetonitrile to trimethylphosphate was done to see if the slight decrease in polarity would increase solubility. This resulted in the appearance of reaction products by thin layer chromatography. Unfortunately, the apparent product could not be isolated from the trimethylphosphate using chromatography.

Figure 3-0-8: Silylation for triazole formation. Alternative protecting group was used for protection of 3' and 5' hydroxyls of 5-bromouridine to overcome solubility issues in synthesis of highly reactive triazole intermediate. This intermediate is desirable for reaction with substituted aminophenols to create secondary amines.
To investigate whether the literature protection groups would overcome the lack of reactivity of the starting material to the triazole, a silyl protection of 5-bromo-2’-deoxyuridine was made (figure 3-0-8). The di-tertbutyl silyl was attached through the combination of the silyl triflate with 5-bromo-2’-deoxyuridine and imidazole in DMF. The triflate leaving group was easily substituted by imidazole forming the reactive N-tert-diterterbutylsilyl imidazole silylating reagent. This activated reagent silylated the 5’ hydroxyl and the imidazole deprotonated the oxygen. The second silylation happens at the neighboring hydroxyl of the nucleoside by the same method. This silylated nucleoside was subjected to the above conditions for triazole activation and again no reaction was observed even upon heating.

Even without this more reactive intermediate, it was possible to accumulate enough of the deprotected secondary amines of these substituted oxygen tricyclic cytosine compounds to perform the ring closure step to complete the substituted nucleosides (figure 3-0-1). The secondary amine of tC was reacted using the literature method for synthesis and this resulted in the desired fluorescent product, but in low yield. The 5’ hydroxyl oxygen provided assistance via attack at the C6 carbon for the leaving of the bromine at the C5 carbon (figure 3-0-9). Fluorine anions are better leaving groups and may take the place of bromine at the C5 position, and then are substituted for by the 2-
Deprotonation of the ring oxygen and reprotonation of the carbohydrate hydroxyl gives the final product.

18-crown-6 ether was added with the starting material under these reaction conditions to increase the dissociation of potassium from the fluoride anions, and this resulted in an apparent increase in yield in a shorter reaction time, but this may have been due to the much smaller scale of the test reaction. Changing the solvent of the reaction from ethanol to diglyme and increasing the temperature led to the conversion of starting material within a few minutes. After purification the yield of the desired compound was low and so the literature method was reconsidered. Yields of around 50% eventually resulted by combining the literature reaction conditions with the laboratory purification conditions discussed below. This was an effective combination for accumulation of

Figure 3-0-11: Ring closure of tC6 nucleosides. Assistance of 5' OH is implicated in the proposed mechanism. Conditions shown are the optimized reagents selected for tolerance of the variety of R groups in this work, hydroxyl oxygen, closing the second ring and creating the conjugated chromophore.\textsuperscript{109}

Figure 3-0-10: Diol chromatography media. Stationary phase used in place of normal silica gel for the purification of nucleosides.
enough compound for further studies to be conducted.

Because of the greater success in the secondary amine formation when using fluoro-aminophenol, the first substituted nucleoside that was synthesized was the 8-F-tC°. Refluxing in ethanol with potassium fluoride and purification via preparatory thin layer chromatography resulted in a fluorescent compound that was confirmed to have the desired mass spectroscopically. Scaling up required longer reaction times even at the same concentration and made purification more difficult. Complete loss of compound and deglycosylation were some of the issues encountered during purification via flash silica gel chromatography. Preparatory thin layer chromatography was not a reasonable means by which to isolate a meaningful amount of compound, so different stationary phases were investigated in the laboratory as will be discussed below. Ultimately a diol media was found to be an efficient way to purify the reaction mixture. This media has an ether chain immobilized on the silica which terminates with a secondary and primary alcohol (figure 3-0-11). Using this less polar stationary phase it is possible to recover more material while removing undesirable impurities. Using diglyme as the solvent and heating the starting material to 120 °C with potassium fluoride and 18-crown-6 affords the desired product in under an hour in reasonable yields.

After the fluorine substituted nucleoside analog was synthesized, it was confirmed that the same conditions could be used to form the tetracyclic tetC°. Purification being an issue at this time, normal phase HPLC was attempted. Method development with cyclohexanes and ethyl acetate was attempted, but the desired product remained difficult to isolate. Preparatory thin layer chromatography was used to develop a standard for
synthesis of the product that was confirmed by mass spectroscopy. Eventually using flash chromatography (dil dil media and dichloromethane/methanol mobile phase) the product could be recovered and the desired mass confirmed spectroscopically. The following is a table (table 4) of the various reaction conditions used for the ring closure of substituted and unsubstituted oxygen tricyclic cytidine analogs.
Table 4: Ring closure of nucleosides. Analog is the name of the substituted product referring to figure 3-0-9. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

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Table 4 Continued: Ring closure of nucleosides: Analog is the name of the substituted product referring to figure 3-4-9. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

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Table 4: Continued. Ring closure of nucleosides. Analog is the name of the substituted product referring to figure 3-0-9. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.
Table 4 Continued: Ring closure of nucleosides. Analog is the name of the substituted product referring to figure 3-0-9. Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.
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3.4.9 Some yields are approximations based on NMR and in the event that no yield is given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.
Table 4 Continued: Ring closure of nucleosides. Analog is the name of the substituted product referring to figure 3-0-9. Some yields are approximations based on NMR and in the event that no yield was given isolated product was not obtained. Some isolated reactions or those for purely production purposes are excluded from this table.

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<td>20min</td>
<td>45min</td>
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Using cesium fluoride as the fluorine source gave similar yields to the reactions with potassium fluoride, but scaling up required multiple rounds of purification due to the large amount of insolubles produced in the reaction. Using this more dissociative fluoride source in dioxane resulted in a complex mixture even after column chromatography. Dioxane is not only a solvent that allows the reaction to be done at higher temperatures, but it also is less likely to solvate the ions necessary for the reaction. NMP was suggested by Dr. Stephen Buchwald in a personal communication regarding amination as a solvent for similar reasons. Ring closure was attempted in these solvents at higher temperature. Reaction at 120 °C gave the desired product, but it was difficult to remove the NMP and there was evidence of some deamination under these conditions. Additionally, scaling up the starting materials reacted under these conditions resulted in significant decreases in yield. Eventually 18-crown-6 in combination with potassium fluoride in ethanol were selected as the best reaction conditions followed by flash chromatography on a diol column to give yields up to 70% for the desired tetracyclic fluorescent nucleoside analog. 18-crown-6 is used to capture the potassium ions in solution to make the fluorine anion a more “naked nucleophile.” In the absence of its counterion, it is more available to be an intermediate for substitution.

Initial attempts to synthesize the 7-Cl-tC° secondary amine under the standard literature conditions resulted in disappearance of the starting material, but this was due to deamination not nucleophilic aromatic substitution. A less concentrated reaction on smaller scale resulted in enough product that the desired mass could be confirmed by mass spectroscopy. Attempting to scale up the dilute reaction resulted in a messy mixture.
of product and chloro-aminophenol, which again indicated deamination, so the switch was made to NMP and cesium fluoride. Using evaporation under ambient conditions to remove the NMP and then flash silica gel chromatography it was possible to isolate the product in fair yield. This evaporation method was less applicable to larger scale reactions, and this resulted in significantly decreased yields for isolable product. DMF was tested for its greater volatility. Purification in the absence of the NMP was not a significantly simpler task. Comparable yields to those from the literature methods were seen with purification via alumina stationary phase following extraction of the crude reaction material with methanol. Returning to the NMP as a reaction solvent because of its assistance in the purification of the compounds, various combinations of evaporation under ambient conditions and purification were attempted. Reactions were even performed in one solvent and then crude mixtures dissolved in smaller quantities of the reaction solvent or an alternative solvent for purification. The greatest success was achieved by flash chromatography on a diol column followed by evaporation under ambient conditions but this did not always result in the removal of the NMP. Ultimately the 18-crown-6 addition to potassium fluoride in diglyme reaction conditions were selected. Using a column with the diol stationary phase for purification followed by hot trituration with a 1:1 mixture of ethanol to water resulted in pure compound. This trituration, although important to achieve high purity, results in the loss of a significant amount of desired product. With the use of a well-conditioned diol column, trituration could be avoided and the yields of 7-Cl-tC° greatly increased.
The 8-Cl-tC\textsuperscript{o} analog in which the chlorine is located at the neighboring position to the analog discussed above was not isolable as the ring closed fluorophore from the parent compound synthetic method. Initial evidence of potential product formation was found by TLC. Following flash silica gel chromatography the initial compound representing potential product was not isolated. Using Cesium fluoride in DMF resulted in evidence of deamination of the starting material. In NMP at even greater temperature, than reaction in alcohol or DMF, and using the same fluoride source the product was produced in fair yield in about an hour. These conditions resulted in product that could be confirmed by mass spectroscopy. Scaling up but maintaining the same concentration it was possible to increase yield of the product, but purification required column chromatography followed by watch-glass evaporation and then further column chromatography. Greater yields were observed in diglyme at the same temperature as the NMP but the purification remained an elaborate combination of chromatography and trituration. The 18-crown-6 ether with potassium fluoride in diglyme conditions were ultimately selected because the starting material concentration could be increased significantly with little loss in reactivity or necessary increase in purification complexity.

When the substituent at this position meta to the second ring nitrogen was a methoxy group instead of chlorine (8-OMe-tC\textsuperscript{o}), it should have been electron donating to the hydroxyl oxygen acting as a nucleophile in this reaction. The 8-OMe-tC\textsuperscript{o} secondary amine was subjected to the conditions discussed for the analogs above as well as some unique reaction environments in attempts to create the fluorescent nucleoside product. Confirmation of the desired mass of the product with mass spectroscopy was achieved
through the examination of the product from the parent compound synthesis method of refluxing in ethanol for three days. Only a portion of this product could be isolated and purification at this stage via flash silica gel chromatography was highly unreliable. Using cesium fluoride in ethanol with this secondary amine analog, an investigation was made into the necessary quality of the solvent for the reaction. No significant difference in reaction performance was seen with either the extra dry or the reagent grade ethanol, both reactions had little product after refluxing 24 hours. A smaller amount of starting material in greater concentration did lead to the desired product with the use of cesium fluoride in refluxing ethanol.

![Figure 3-0-12: Silver tetrafluoroborate ring closure. Alternative ion to the fluoride used in literature synthesis of ring closed tricyclic cytosine analogs.](image)

As with the nucleobase analogs, silver tetrafluoroborate with its weakly coordinating anion was used as a substitute for potassium fluoride (figure 3-0-12). A reaction initially attempted in dichloroethane at room temperature to which ethanol was added and heat applied failed to result in reaction of the starting material. Beginning with ethanol at 85 °C did not make a difference; there was no reaction under these conditions either. Performing the reaction over molecular sieves to ensure that interference from water was minimized was of no assistance, there continued to be no reaction of the starting material. Adding an additional equivalent of the tetrafluoroborate salt was a last
effort to encourage reaction under these conditions but the starting material remained unreacted.

Cesium fluoride in dioxane resulted in no product formation but this fluoride source in NMP was an effective means of synthesizing the desired 8-OMe-tC\textsuperscript{6} on small scale. In larger quantities, the removal of NMP was again a challenge. This challenge was compounded by the tendency of the methoxy secondary amine to produce two fluorescent products. This is possibly the result of anomeric isomerization, but the mass spectral analysis of these two compounds was inconclusive. Switching the solvent to DMF requires a decrease in the reaction temperature from 120 °C to 100 °C and this change could be part of the reason that the reaction performance is decreased. Isolated amounts from larger scale reactions were greater from reactions with DMF, but the better reaction performance in NMP made it an attractive solvent to revisit. In one larger scale reaction, calcium carbonate was added to clear away the fluorine ions that were hypothesized to be the reason behind the difficulties in purification, but this did not improve isolation. Multiple diol chromatographic exercises did result in the accumulation of some fluorescent nucleoside. The 18-crown-6 ether conditions with potassium fluoride in diglyme at 120 °C were selected for the synthesis of the product. In the likely event that multiple chromatographic runs are necessary to purify the compound, evaporation onto celite was shown to lead to greater recovery of product than using alumina to load material for diol chromatography. Occasionally recrystallization from a 1:1 mixture of ethanol and water is a useful purification method as well but the drawbacks of this technique was described above.
The methoxy secondary amine where the ether is in the electron withdrawing position meta to this nucleophilic hydroxyl was not subjected to the ring closure conditions of the parent compound, only under more optimized conditions due to its general scarcity. On small scale a good yield of the 7-OMe-tC\textsuperscript{o} fluorescent nucleoside was achieved from the secondary amine with cesium fluoride in NMP. Larger scales with which a mass spectroscopy sample could be afforded were not successful until the solvent was changed from NMP to DMF. This material was stubbornly difficult to isolate until the diol columns began to be used for purification In diglyme with 18-crown-6 ether and potassium fluoride, the secondary amine of 7-OMe-tCo affords the greatest amount of the desired product from reactions of the largest scale.

Individual reaction conditions may outperform those that have been selected for the synthesis of these substituted tC\textsuperscript{o}s, but the conditions selected are the only ones that are reliable for the production of the entire small library(figure 3-0-10). These conditions made it possible to produce the compound for florescence investigation and for enzyme tolerance studies as will be discussed in the remainder of the document. Hopefully an improved synthetic route to these compounds will be one day developed.
Oxygen Tricyclic Cytidine Analogs Experimental:

5-bromo-2'-deoxyuridine. 2'-deoxyuridine (500 mg, 2.19 mmol) was dissolved in dry DMF (5 mL) under N₂. 1,3-dibromo-5,5-dimethylhydantoin (345 mg, 1.21 mmol) was added and the reaction stirred at room temperature. After 30 minutes the reaction was evaporated and then co-evaporated with acetonitrile to give product (463 mg, 1.51 mmol) in 69% yield. ¹HNMR (CD₃SO) δ 11.78-11.74 (m, 1H), 8.38 (s, 1H), 6.10 (t, J=6.5 Hz, 1H), 5.22 (d, J=4.4 Hz, 1H), 5.14 (t, J=4.9 Hz, 1H), 4.28-4.20 (m, 1H), 3.80 (q, J=3.3 Hz, 1H), 3.67-3.53 (m, 2H), 2.20-2.05 (m, 2H).

3',5'-diacetyl-5-bromo-2'deoxyuridine. 2'-deoxy-5-bromouridine (463 mg, 1.51 mmol) was dissolved in pyridine (4.6 mL) under N₂. Acetic anhydride (569 μL, 6.02 mmol) was added and the reaction maintained at room temperature. 18 hours later the reaction was diluted with ethyl acetate (15 mL) washed with 0.121 M HCl (15 mL), dried and evaporated. Purification via flash silica gel chromatography (CH₃OH in CH₂Cl₂) gave the product (500 mg, 1.28 mmol) in 85% yield. ¹HNMR (CD₃OD) δ 8.07 (s, 1H), 6.25 (dd, J=8.0, 5.9 Hz, 1H), 5.30 (dt, J=6.7, 2.4Hz, 1H), 4.45-4.40 (m, 1H), 4.37-4.32 (m, 2H), 2.52 (ddd, J=14.5, 6.0, 2.4 Hz, 1H), 2.42 (ddd, J=14.5, 8.1, 6.6Hz, 1H), (2.18 (s,
3H), 2.12 (S, 3H).$^{13}$CNMR ((CD$_3$)$_2$SO) 171.00, 170.94, 159.97, 150.61, 140.87, 97.21, 85.96, 82.42, 74.72, 64.50, 36.96, 21.69, 21.57.

*General Procedures for formation of 3’,5’-diacetyl-5-bromo-N4-(2-hydroxyphenyl)-2’deoxyxytidines.*

Method A: 3’,5’-diacetyl-5-bromo-2’deoxyuradine (850 mg, 2.17 mmol) is combined with 4-dimethylaminopyridine (13 mg, 0.106 mmol) under N$_2$. This was dissolved in dichloromethane (10 mL) and triethylamine (1.2 mL, 8.7 mmol) was added followed by 2-mesitylenesulfonyl chloride (950 mg, 4.34 mmol) and the reaction stirred at room temperature. After 19 hours, 1,8-diazabicycloundec-7-ene (650 μL, 4.35 mmol) was added followed by aminophenol (4.35 mmol) and the reaction continued at room temperature. After reaction appeared complete via thin layer chromatography, the mixture was evaporated. The crude residue was suspended between ethyl acetate and sodium bicarbonate, then organics were dried and evaporated. Purification via silica gel flash chromatography (CH$_3$OH in CH$_2$Cl$_2$) gives a semi-crude mixture that is subjected to following reaction conditions.

Method B: N-methyylimidazole (410 μL, 5.11 mmol) was dissolved in dry acetonitrile (1.2 mL) under N$_2$. After cooling in a calcium chloride ice bath, phosphorous oxychloride (140 μL, 1.53 mmol) in dry acetonitrile (1.2 mL) was added to the reaction dropwise. 3’,5’-diacetyl-5-bromo-2’deoxyuradine (200 mg, 0.511 mmol) which had been evaporated two times from dry acetonitrile was added to the reaction in dry acetonitrile (1.5 mL) and the reaction was warmed to room temperature. Aminophenol (5.11 mmol) which had been evaporated from dry pyridine was added to the reaction in dry pyridine (2
mL) after it had been at room temperature 15 minutes. Triethylamine (700 μL, 5.07 mmol) was added to the reaction and it was kept at room temperature then cooled to 5 degrees. Dilution with water and evaporation of the reaction mixture was followed by co-evaporation with ethyl acetate. Residue dissolved in chloroform was washed with sodium bicarbonate and then brine, dried and evaporated. Purification via flash silica gel chromatography (EtOAc in Cyclohexanes) gives semicrude product used for further synthetic application without further purification.

Method C: Dry carbon tetrachloride (4 mL) was mixed with dry dichloromethane (4 mL) under N₂. Triphenylphosphine (639 mg, 2.44 mmol) was added and the reaction was stirred at room temperature 15 minutes until yellow in color. 3’,5’-diacetyl-5-bromo-2’-deoxyuridine 4 (500 mg, 1.28 mmol) was added to the reaction which was then heated to 44 °C. After 5 hours the reaction was cooled to room temperature and then to 0 °C. 2-aminophenol (1.278 mmol) with DBU (190 μL, 1.278 mmol) in dry dichloromethane (1 ml) was added to the reaction and stirred at 0 °C until thin layer chromatography showed deamination of the product. The reaction was evaporated and purified via flash chromatography (CH₃OH in CH₂Cl₂) to yield semi-crude product that was subjected to the next synthetic sequence.

Method D: Dry carbon tetrachloride (4 mL) was dissolved in dry dichloromethane (4 mL) under N₂. Triphenylphosphine (639 mg, 2.44 mmol) was added and the reaction was stirred at room temperature 15 minutes until yellow in color. 3’,5’-diacetyl-5-bromo-2’-deoxyuridine 4 (500 mg, 1.28 mmol) was added to the reaction which was then heated to 44°C. After 5 hours 2-aminophenol (1.278 mmol) with DBU (190 μL, 1.278 mmol) in
dry dichloromethane (1 ml) was added to the reaction and stirred at 44 °C until thin layer chromatography showed deamination of the product. The reaction was evaporated and purified via flash chromatography (CH$_3$OH in CH$_2$Cl$_2$) to yield semi-crude product that was subjected to the next synthetic sequence.

3',5'-diacetyl-5-bromo-N4-(2-hydroxyphenyl)-2'-deoxycytidine. Synthesized in crude via methods A B and C from 2-aminophenol. $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 10.23 (s, 1H), 8.92 (s, 1H), 8.13 (dd, J=8.0, 1.6 Hz, 1H), 8.10 (s, 1H), 7.02 (ddd, J=8.8, 6.3, 1.6 Hz, 1H), 6.93 (dd, J=8.1, 1.5 Hz, 1H), 6.89 – 6.80 (m, 1H), 6.15 (dd, J=7.7, 6.3 Hz, 1H), 5.19 (dq, J=7.3, 4.3, 3.6 Hz, 1H), 4.29 (dd, J=4.6, 1.5 Hz, 2H), 4.24 (td, J=4.5, 2.8 Hz, 1H), 2.44 – 2.35 (m, 2H), 2.09 (s, 3H), 2.07 (s, 3H).

3',5'-diacetyl-5-bromo-N4-(2-hydroxynaphtol)-2'-deoxycytidine. Synthesized via methods A and C from 2-aminonapthol. $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 11.00 (s, 0H), 8.85 (s, 1H), 8.17 (s, 1H), 7.77 – 7.73 (m, 1H), 7.71 – 7.67 (m, 1H), 7.36 (ddd, J=8.1, 6.9, 1.3 Hz, 1H), 7.33 – 7.28 (m, 1H), 7.26 (s, 1H), 6.18 (dd, J=7.6, 6.3 Hz, 1H), 5.22 (dt, J=6.2, 2.9 Hz, 1H).
Hz, 1H), 4.31 (dd, J=4.5, 2.2 Hz, 2H), 4.26 (tt, J=4.4, 2.3 Hz, 1H), 2.46–2.39 (m, 2H), 2.09 (s, 3H), 2.08 (s, 3H).

3’,5’-diacetyl-5-bromo-N4-(2-hydroxy-4-chlorophenyl)-2’-deoxycytidine.

Synthesized in crude via methods A, B, and C from 2-amino-5-chlorophenol. $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 8.40 (s, 1H), 8.11 (s, 1H), 8.10 – 8.06 (m, 1H), 6.94 (t, J=2.3 Hz, 1H), 6.93 – 6.88 (m, 1H), 6.14 (dd, J=7.8, 6.3 Hz, 1H), 5.19 (dt, J=6.3, 2.8 Hz, 1H), 4.29 (dd, J=4.6, 1.7 Hz, 2H), 4.23 (td, J=4.5, 2.7 Hz, 1H), 2.46 – 2.37 (m, 2H), 2.08 (s, 3H), 2.07 (s, 3H), 1.23 (s, 1H).

3’,5’-diacetyl-5-bromo-N4-(2-hydroxy-4-chlorophenyl)-2’-deoxycytidine.

Synthesized via method D from 2-amino-4-chlorophenol. $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 10.69 (s, 1H), 8.32 (d, J=2.7 Hz, 1H), 8.14 (s, 1H), 7.06 (dd, J=8.6, 2.7 Hz, 1H), 6.93 (d, J=8.6 Hz, 1H), 6.15 (t, J=6.9 Hz, 1H), 5.20 (dt, J=6.2, 2.9 Hz, 1H), 4.29 (dt, J=4.5, 2.1 Hz, 2H), 4.27 – 4.24 (m, 1H), 2.45 – 2.39 (m, 2H), 2.08 (s, 3H), 2.07 (s, 3H).

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3',5'-diacetyl-5-bromo-N4-(2-hydroxy-5-fluorophenyl)-2'-deoxycytidine.

Synthesized in semicrude form and used for further synthetic application without further purification via methods A and C from 2-amino-4-fluorophenol. $^1$H NMR ((CD$_3$)$_2$SO) δ 10.41 (s, 1H), 8.22 (dd, J=10.9, 3.0 Hz, 1H), 8.15 (s, 1H), 6.91 (dd, J=8.9, 5.4 Hz, 1H), 6.84 (td, J=8.6, 3.1 Hz, 1H), 6.15 (dd, J=7.6, 6.3 Hz, 1H), 5.20 (dt, J=6.2, 2.9 Hz, 1H), 4.29 (dt, J=5.4, 2.5 Hz, 2H), 4.25 (tt, J=4.6, 2.0 Hz, 1H), 2.46 – 2.37 (m, 2H), 2.08 (s, 3H), 2.07 (d, J=3.1 Hz, 3H).

3',5'-diacetyl-5-bromo-N4-(2-hydroxy-5-methoxyphenyl)-2'-deoxycytidine.

Synthesized in semicrude form and used for further synthetic application without further purification via methods A and C from 2-amino-4-methoxyphenol. $^1$H NMR ((CD$_3$)$_2$SO) δ 9.83 (s, 1H), 8.11 (s, 1H), 7.94 (d, J=3.1 Hz, 1H), 6.84 (d, J=8.8 Hz, 1H), 6.61 (dd, J=8.8, 3.0 Hz, 1H), 6.15 (dd, J=7.7, 6.3 Hz, 1H), 5.20 (dt, J=6.2, 2.9 Hz, 1H), 4.29 (dd, J=4.5, 2.0 Hz, 2H), 4.26 – 4.22 (m, 1H), 3.68 (s, 3H), 2.45 – 2.35 (m, 2H), 2.09 (s, 3H), 2.07 (s, 3H).
3',5'-diacetyl-5-bromo-N4-(2-hydroxy-4-methoxyphenyl)-2'-deoxycytidine.

Synthesized in semicrude form and used for further synthetic application without further purification via method C from 2-amino-5-methoxyphenol. $^1$H NMR ((CD$_3$)$_2$SO) δ 8.04 (s, 1H), 7.77 (d, J=8.8 Hz, 1H), 6.49 (d, J=2.7 Hz, 1H), 6.43 (dd, J=8.9, 2.8 Hz, 1H), 6.14 (dd, J=8.0, 6.2 Hz, 1H), 5.18 (dt, J=6.4, 2.9 Hz, 1H), 4.28 (dd, J=4.4, 1.5 Hz, 2H), 4.22 (td, J=4.5, 2.8 Hz, 1H), 3.71 (s, 3H), 2.41 – 2.32 (m, 2H), 2.09 (s, 3H), 2.06 (s, 3H).

General Procedures for formation of 5-bromo-N4-(2-hydroxyphenyl)-2’deoxyctydines.

Method E: 3’,5’-diacetyl-5-bromo-N$^4$-(2-hydroxyphenyl)-2’deoxyctydine (265 mg, 0.512 mmol) was dissolved in HPLC grade methanol (3 mL) under N$_2$. Sodium methoxide (380 μL, 2.05 mmol) 30% solution in methanol was added to the reaction and stirred at room temperature until thin layer chromatography revealed reaction completion. Acetic acid (117 μL, 2.05 mmol) was added to the reaction and it was evaporated. Purification via flash silica gel chromatography (CH$_3$OH in CH$_2$Cl$_2$) to give the product.
5-bromo-N4-(2-hydroxyphenyl)-2'-deoxycytidine. Synthesized via methods E from 3',5'-diacetyl-5-bromo-N4-(2-hydroxyphenyl))-2'-deoxycytidine. Yields ranging from 12-76% over two steps. $^1$HNMR (CD$_3$OD) $\delta$ 8.58 (s, 1H), 8.47 (dd, J=8.1, 1.6 Hz, 1H), 7.01 (td, J=7.7, 1.6 Hz, 1H), 6.93-6.85 (m, 2H), 6.35 (t, J=6.1 Hz, 1H), 4.59 (s, 1H), 4.42 (dt, J=6.3, 4.3 Hz, 1H), 4.00 (q, J=3.3 Hz, 1H), 3.90 (dd, J=12.1, 2.9 Hz, 1H), 3.79 (dd, J=12.1, 3.3 Hz, 1H), 2.470 (dd, J=13.7, 6.2, 4.5 Hz, 1H), 2.23 (dt, J=13.7, 6.2 Hz, 1H). $^{13}$CNMR ((CD$_3$)$_2$SO) 157.00, 153.16, 148.03, 141.86, 125.99, 125.04, 122.09, 119.00, 114.92, 87.56, 85.73, 69.66, 60.64, 40.80, 30.69.

5-bromo-N4-(2-hydroxynaphtol)-2'-deoxycytidine. Synthesized via method E from 3',5'-diacetyl-5-bromo-N4-(2-hydroxynaphthol))-2'-deoxycytidine. Yields ranging from 5-65% over two steps. $^1$HNMR ((CD$_3$)$_2$SO) $\delta 11.02$ (s, 1H), 8.68-8.59 (m, 1H), 8.56 (s, 1H), 7.78-7.73 (m, 1H), 7.74-7.69 (m, 1H), 7.37 (ddd, J=8.1, 6.9, 1.4 Hz, 1H), 7.32 (ddd, J=8.1, 6.8, 1.4 Hz, 1H), 7.28 (s, 1H), 6.15 (t, J=6.2 Hz, 1H), 5.29 (d, J=4.4 Hz, 1H), 5.24 (t, J=4.9 Hz, 1H), 4.27 (dq, J=7.9, 4.1 Hz, 1H), 4.13 (q, J=5.3 Hz, 1H), 3.74-3.67 (m,
1H) 3.62 (dt, J=11.9, 4.1 Hz, 1H), 2.26 (ddd, J=13.2, 6.2, 4.1 Hz, 1H), 2.13 (dt, J=13.0, 6.3 Hz, 1H). MS (ESI) calcd. for C₁₉H₁₉BrN₅O₅ 448.1, found 448.1 (MH+).

5-bromo-N₄-(2-hydroxy-4-chlorophenyl)-2’-deoxycytidine. Synthesized via methods E from 3’,5’-diacetyl-5-bromo-N₄-(2-hydroxy-4-chlorophenyl))-2’-deoxycytidine. Yields ranging from 2.5-82%. ¹HNMR ((CD₃)₂SO) δ 10.94 (s, 1H), 8.49 (s, 1H), 8.36 (s, 1H), 8.18 (d, J=8.6 Hz, 1H) 6.98-6.86 (m, 2H), 6.11 (t, J=6.2 Hz, 1H), 5.26 (d, J=4.2 Hz, 1H), 5.22 (d, J=5.2 Hz, 1H), 4.25 (s, 1H), 3.83 (q, J=3.4 Hz, 1H), 3.68 (dt, J=11.9, 3.6 Hz, 1H) 3.60 (dt, J=12.1, 3.6 Hz, 1H), 2.22 (ddd, J=13.2, 6.2, 4.1 Hz, 1H), 2.09 (dt, J=13.0, 6.3 Hz, 1H). MS (ESI) calcd. for C₁₅H₁₅BrClN₅O₅ 434.0, found 434.0 (MH+).

5-bromo-N₄-(2-hydroxy-5-chlorophenyl)-2’-deoxycytidine. Synthesized via method E from 3’,5’-diacetyl-5-bromo-N₄-(2-hydroxy-5-chlorophenyl))-2’-deoxycytidine. Yields from 18-41%. ¹HNMR ((CD₃)₂SO) δ 10.72 (s, 1H), 8.53 (s, 1H), 8.39 (s, 1H)*, 8.33 (d, J=10.1 Hz, 1H) 7.06 (dd, J=8.6, 2.6 Hz, 1H), 6.95 (d, J=8.6 Hz, 1H), 6.11 (t, J=6.2 Hz, 1H), 5.28 (d, J=4.4 Hz, 1H), 5.22 (t, J=5.0 Hz, 1H), 4.34-4.22 (m,
1H), 3.85 (q, J=3.4 Hz, 1H), 3.69 (ddd, J=11.9, 5.0, 3.3 Hz, 1H) 3.60 (ddd, J=11.9, 4.8,
3.3 Hz, 1H), 2.24 (ddd, J=13.2, 6.2, 4.2 Hz, 1H), 2.10 (dt, J=13.0, 6.2 Hz, 1H). MS (ESI)
calcd. for NaC_{15}H_{14}BrClN_{3}O_{5} 456.0, found 456.0 (MNa+).

5-bromo-N4-(2-hydroxy-5-fluorophenyl)-2'-deoxycytidine. Synthesized via
method E from 3',5'-diacetyl-5-bromo-N4-(2-hydroxy-5-fluorophenyl)-2'-
deoxycytidine. Yields from 6-31%.

\[ ^1{H}NMR \ (\text{CD}_3\text{SO}) \delta 10.45 \ (s, 1H), 8.54 \ (s, 1H), 8.32 \ (s, 1H), 8.28 \ (dd, J=10.9, 3.1 Hz, 1H) \]
\[ 6.92 \ (dd, J=8.9, 5.4 Hz, 1H), 6.84 \ (td, J=8.5, 3.2 Hz, 1H), 6.12 \ (t, J=6.2 Hz, 1H), 5.28 \ (d, J=4.4 Hz, 1H), 5.26-5.17 \ (m, 1H), 4.26 \ (dq, J=7.4, 4.1 Hz, 1H), 3.85 \ (q, J=3.4 Hz, 1H), 3.72-3.65 \ (m, 1H), 3.61 \ (ddt, J=11.9, 7.8, 4.1 Hz, 1H), 2.24 \ (ddd, J=13.3, 6.2, 4.2 Hz, 1H), 2.18-2.05 \ (m, 1H). MS (ESI) calcd. for NaC_{15}H_{15}BrFNO_{5} 438.0, found 438.0 (MNa+).

5-bromo-N4-(2-hydroxy-5-methoxyphenyl)-2'-deoxycytidine. Synthesized via
method E from 3',5'-diacetyl-5-bromo-N4-(2-hydroxy-5-methoxyphenyl)-2'-
deoxycytidine. Yields ranging from 3-88%.

\[ ^1{H}NMR \ (\text{CD}_3\text{SO}) \delta 9.86 \ (s, 1H), 8.50 \ (s, 1H), 8.32 \ (s, 1H), 8.00 \ (d, J=3.0 Hz, 1H) \]
\[ 6.85 \ (d, J=8.7 Hz, 1H), 6.61 \ (dd, J=8.8, 3.1 Hz, 1H), 5.90 \ (d, J=4.8 Hz, 2H), 4.83 \ (t, J=4.8 Hz, 2H), 3.90 \ (s, 3H), 3.78 \ (s, 3H). MS (ESI) calcd. for NaC_{15}H_{15}BrNO_{5} 424.0, found 424.0 (MNa+). \]
1H), 6.12 (t, J=6.3 Hz, 1H), 5.27 (d, J=4.4 Hz, 1H), 5.22 (t, J=5.0 Hz, 1H), 4.25 (dt, J=7.9, 4.0 Hz, 1H), 3.84 (q, J=3.4 Hz, 1H), 3.70 (s, 3H), 3.70-3.65 (m, 1H), 3.60 (dt, J=12.0, 3.8 Hz, 1H), 2.23 (ddd, J=13.3, 6.2, 4.1 Hz, 1H), 2.10 (dt, J=13.0, 6.2 Hz, 1H). MS (ESI) calcd. for C_{16}H_{18}BrN_{3}O_{6} 430.0, found 430.1 (MH+).

5-bromo-N4-(2-hydroxy-4-methoxyphenyl)-2'-deoxycytidine. Synthesized via method E from 3’,5’-diacetyl-5-bromo-N4-(2-hydroxy-4-methoxyphenyl)-2’-deoxycytidine. Yields ranging from 2-56%. \textsuperscript{1}HMR ((CD_{3})_{2}SO) δ 8.42 (d, J= 0.7 Hz, 1H), 8.34 (s, 1H), 7.85 (d, J = 8.8 Hz, 1H), 6.50 (d, J=2.8 Hz, 1H) 6.44 (dd, J=8.9, 2.8 Hz, 1H), 6.12 (q, J=6.3 Hz, 1H), 5.23 (d, J=30.8 Hz, 2H), 4.25 (dq, J=7.4, 4.1 Hz, 1H), 3.82 (dq, J=6.3, 3.3 Hz, 1H), 3.66 (td, J=11.9, 3.3 Hz, 1H), 3.59 (dt, J=9.1, 2.8 Hz, 1H), 3.37 (s, 3H), 2.24-2.15 (m, 1H), 2.14-2.04 (m, 1H). MS (ESI) calcd. for C_{16}H_{18}BrN_{3}O_{6} 430.0, found 430.1 (MH+).

General Methods for the synthesis of 1,3-diaza-2-oxophenoxazine 2’-deoxy-β-D-ribofuranosides.

Method F: 5-bromo-N^4-(2-hydroxyphenyl)-2’-deoxycytidine (653 mg, 1.64 mmol) was dissolved in ethanol (13 mL) under N\textsubscript{2}. Potassium fluoride (953 mg, 16.4 mmol) was added and the reaction was heated to reflux. After 3 days the starting material appears completely converted by thin layer chromatography and the reaction is cooled to
room temperature and evaporated. Purification off of celite via diol chromatography (CH$_3$OH in CH$_2$Cl$_2$) yields the product.

Method G: 5-bromo-N$^4$-(2-hydroxyphenyl)-2'-deoxycytidine (500 mg, 0.967 mmol) was combined with 18-crown-6 (1.28 g, 4.83 mmol) under N$_2$. This was dissolved in dry diglyme (4 mL) and heated to 120 °C to dissolve. Potassium fluoride (281 mg, 4.83 mmol) was added and the reaction was heated to 120 °C and stirred until thin layer chromatography revealed complete conversion of the starting material. Cooling to room temperature followed by purification of the reaction via diol chromatography (CH$_3$OH in CH$_2$Cl$_2$) to yield the product.

Method H: 5-bromo-N$^4$-(2-hydroxyphenyl)-2'-deoxycytidine (150 mg, 0.277 mmol) was combined with cesium fluoride (421 mg, 2.77 mmol) under N$_2$. This was dissolved in dry diglyme$^a$ (5 mL) or dry NMP$^b$ (6 mL) or dry DMF$^c$ (7.5 mL) and heated to 120 °C. The reaction was stirred until thin layer chromatography revealed complete conversion of the starting material. Cooling to room temperature followed by purification of the reaction via diol chromatography (CH$_3$OH in CH$_2$Cl$_2$) to yield the product.

8-fluoro-1,3-diaza-2-oxophenoxazine 2′-deoxy-β-D-ribofuranoside. Synthesized via methods F and G from 5-bromo-N4-(2-hydroxy-5-fluorophenyl)-2′-deoxycytidine. Yields up to 25%. $^1$HNMR ((CD$_3$)$_2$SO) δ 10.88 (s, 1H), 7.51 (s, 1H), 6.80 (dd, J=8.9, 5.0 Hz, 1H), 6.66 (td, J=8.6, 3.0 Hz, 1H), 6.60 (dd, J=9.4, 3.0 Hz, 1H) 6.12 (dd, J=7.4, 6.0 Hz, 1H)
Hz, 1H), 5.24 (s, 1H), 5.12 (s, 1H), 4.22 (dt, J=6.2, 3.1 Hz, 1H), 3.77 (q, J=3.3, 1H), 3.59 (td, J=10.5, 9.1, 3.6 Hz, 2H), 2.10-1.97 (m, 2H). $^{13}$CNMR ((CD$_3$)$_2$SO) 158.80, 156.91, 153.13, 152.16, 138.90, 127.15, 115.60, 115.52, 109.23, 109.04, 87.29, 84.73, 70.27, 61.13. AMM (ESI) calcd. for C$_{15}$H$_{14}$N$_3$O$_5$F 335.09, found 336.10 (MH+).

1,3-diaza-benzo(h)2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside. Synthesized via methods F, G and H$^b$ from 5-bromo-N$^4$-(2-hydroxynaphthol)-2'-deoxycytidine. Yields from 3-71%. $^1$HNMR ((CD$_3$)$_2$SO) δ 8.56 (s, 1H), 7.72 (s, 1H), 7.70-7.63 (m, 2H), 7.38-7.28 (m, 2H), 7.27 (s, 1H), 7.21 (s, 1H), 6.16 (s, 1H), 5.41 (s, 2H), 4.28 (s, 1H), 3.79 (s, 1H), 3.63 (t, J=4.0 Hz, 2H), 2.10 (s, 2H). $^{13}$CNMR ((CD$_3$)$_2$SO) 172.77, 165.20, 152.45, 142.21, 130.39, 130.29, 126.63, 126.59, 126.42, 125.13, 124.94, 121.52, 113.28, 110.34, 87.37, 84.83, 72.43, 60.97. AMM (ESI) calcd. for C$_{19}$H$_{17}$N$_3$O$_5$ 367.12, found 368.12 (MH+).

7-chloro-1,3-diaza-2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside. Synthesized via methods F, G, H$^b$ and H$^c$ from 5-bromo-N4-(2-hydroxy-4-chlorophenyl)-2'
deoxycytidine. Yields ranging up to 60%. $^1$HNMR ((CD$_3$)$_2$SO) $\delta$ 10.89 (s, 1H), 7.48 (s, 1H), 6.98-6.86 (m, 2H), 6.77 (d, J=8.4 Hz, 1H), 6.13 (dd, J=7.4, 6.1 Hz, 1H), 5.24 (s, 1H), 5.13 (s, 1H), 4.23 (dt, J=6.1, 3.1 Hz, 1H), 3.78 (q, J=3.3, 1H), 3.64-3.56 (m, 2H), 2.12-1.97 (m, 2H). $^{13}$CNMR ((CD$_3$)$_2$SO) 153.23, 152.37, 143.32, 134.76, 127.05, 126.51, 123.23, 120.21, 119.13, 114.78, 87.25, 84.70, 70.44, 61.25, 48.61. AMM (ESI) calcd. for C$_{15}$H$_{14}$N$_3$O$_5$Cl 351.06, found 703.13 (2MH+).

8-chloro-1,3-diaza-2-oxophenoxazine 2'-deoxy-$\beta$-D-ribofuranoside. Synthesized via methods F, G, H$^a$ and H$^b$ from 5-bromo-N4-(2-hydroxy-5-chloro-phenyl)-2'-deoxycytidine. Yields ranging up to 75%. $^1$HNMR ((CD$_3$)$_2$SO) $\delta$ 7.48 (s, 1H), 6.83 (dd, J=8.5, 2.5 Hz, 1H), 6.80-6.68 (m, 2H), 6.12 (dd, J=7.3, 6.0 Hz, 1H), 4.24 (dt, J=6.3, 3.3 Hz, 1H), 3.76 (q, J=3.3 Hz, 1H), 3.58 (t, J=3.6 Hz, 2H), 2.10-1.98 (m, 2H). $^{13}$CNMR ((CD$_3$)$_2$SO) 172.76, 153.65, 152.38, 141.66, 127.21, 126.71, 122.53, 119.98, 117.55, 115.93, 87.22, 84.66, 70.21, 61.11. AMM (ESI) calcd. for C$_{15}$H$_{14}$N$_3$O$_5$Cl 351.06, found 703.13 (2MH+).
8-methoxy-1,3-diaza-2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside.

Synthesized via methods F, G, H\textsuperscript{b} and H\textsuperscript{c} from 5-bromo-N4-(2-hydroxy-5-methoxyphenyl)-2'-deoxycytidine. Yields ranging up to 51%.

\(^1\)HNMR ((CD\(_3\)\(_2\)SO) \(\delta\)

10.63 (s, 1H), 7.56 (s, 1H), 6.75 (d, J=8.6 Hz, 1H), 6.45-6.37 (m, 2H), 6.14 (dd, J=7.4, 6.0 Hz, 1H), 5.24 (s, 1H), 5.12 (s, 1H), 4.24 (dt, J=6.2, 3.1 Hz, 1H), 3.79 (q, J=3.3, 1H), 3.68 (s, 3H), 3.59 (dd, J=9.7, 3.5 Hz, 2H), 2.09 (ddd, J=13.1, 6.0, 3.2 Hz, 1H), 2.02 (ddd, J=13.2, 7.5, 5.9 Hz, 1H).

\(^{13}\)CNMR ((CD\(_3\)\(_2\)SO) 172.86, 153.75, 152.43, 141.69, 127.26, 126.72, 122.52, 119.97, 117.62, 115.93, 87.21, 84.68, 70.27, 69.54, 61.16, 60.34. AMM (ESI) calcd. for C\(_{16}\)H\(_{17}\)N\(_3\)O\(_6\) 347.11, found 348.12 (MH+)  

7-methoxy-1,3-diaza-2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside.

Synthesized via methods G, H\textsuperscript{b} and H\textsuperscript{c} from 5-bromo-N4-(2-hydroxy-4-methoxyphenyl)-2'-deoxycytidine. Yields ranging up to 75%.

\(^1\)HNMR ((CD\(_3\)\(_2\)SO) \(\delta\)

7.46 (s, 1H), 6.49 (d, J=8.6 Hz, 1H), 6.45 (dd, J=8.6, 2.7 Hz, 1H), 6.45 (d, J=2.7 Hz, 1H), 6.15 (t, J=6.8, 1H), 5.25 (s, 1H), 4.23 (d, J=5.3 Hz, 1H), 3.78 (q, J=323, 1H), 3.69 (s, 3H), 3.58 (s, 2H), 2.11-1.96 (m, 2H). AMM (ESI) calcd. for C\(_{16}\)H\(_{17}\)N\(_3\)O\(_6\) 347.11, found 348.12 (MH+).
Synthesis of Other Oxygen Tricyclic Cytidine Analogs

A nitro tricyclic cytosine analog has been previously reported; it was designed for its’ FRET acceptor abilities. In this work, a new synthesis was designed for a dinitro-substituted tC analog has been synthesized with the goal of studying its photophysical properties. This dinitro-tC is synthesized in one step through the reaction of difluoro-dinitrobenzene with 5-hydroxycytidine (figure 3-0-13). In DMF and with triethylamine as base, the reaction works without the protection of the 3’ and 5’ hydroxyl groups. This reaction operates through double nucleophilic aromatic substitution reactions between the amine and the phenol of the nucleoside and 1,2-difluoro-4,5-dinitrobenzene. This double substitution results in a deep red compound that has absorptive but not fluorescence properties as will be discussed in the following chapter.

![Figure 3-0-13: Dinitro tC synthesis. Dinitro tricyclic cytosine analog 7,8-NO2-tC that was synthesized in one step from hydroxy cytidine by reaction with difluoro-dinitro-benzene.]

This dinitro-tC 7,8-NO2-tC analog was used as a precursor for the synthesis of a diamino analog because of the desire for amine functionality to direct photophysics as discussed in the previous chapter. Palladium on charcoal was combined with ammonium formate in methanol in initial attempts to reduce the nitro groups to amines. As was discussed for the hydrogenolysis of benzyl protecting groups previously, the ammonium formate serves as an in situ hydrogen source. The hydrogen serves as a source for protons which are combined with two electrons from the palladium to reduce the nitro to the
dinitro through loss of water. An additional two protons with an additional two electrons reduce the nitroso to the hydroxylamine. The final two protons and two electrons and the loss of water afford the amine from the hydroxylamine.

When the above reaction conditions failed to produce a reaction, hydrazine monohydrate was substituted for the ammonium formate and the solvent was switched to ethanol and heated at 95 °C (figure 3-0-14). Reduction of aromatic nitro compounds is known to proceed through the short lived reducing agent diimide. This reaction formed a deeper red color and evidence of two new compounds were seen but they were not recovered in purification. Repeating these conditions at 70 °C yielded some potential product which was purified via preparatory thin layer chromatography. This product was dissolved in pyridine with acetic anhydride to acetylate the amines. Complete conversion of the starting material was seen but proton NMR revealed that likely what had occurred was deglycosylation. An in-situ acetylation was attempted following the reduction of the dinitro under the above conditions but the results of this experiment were inconclusive as the product showed evidence of the sugar portion of the product but not all of the expected aromatic hydrogens.

To have the option to avoid this glycosidic lability, and forgo expense of the starting material the synthesis was attempted from cytosine. Elbs persulfate oxidation is
useful in the hydroxyl substitution of the nucleobase. Reacting the nucleobase with potassium persulfate in potassium hydroxide followed by acidification with hydrochloric acid generates the sulfate intermediate (figure 3-0-15). Oxidation of the base is achieved through the deprotonation of the N1 nitrogen by hydroxide and the resonance of the nucleobase to the C5 carbanion. These electrons are used to attack the peroxide oxygen of the peroxodisulfide expelling the other sulfate. Potassium provides the counterion for the sulfate, and the deprotonation of C5 is followed by resonance back to the nitrogen anion which is then protonated. During workup the N3 nitrogen is protonated to generate the zwiterionic intermediate.

![Figure 3-0-15: Hydroxy-cytosine synthesis. Elbs persulfate oxidation synthesis was implemented in conversion of cytosine to starting material for the synthesis of dinitro tricyclic cytosine nucleobase.](image)

Reaction of this sulfate intermediate with additional hydrochloric acid followed by neutralization yielded 5-hydroxycytosine. Hydrolysis of the sulfate is achieved through the protonation of the sulfate oxygen and then substitution at C5 by water to expel this leaving group. Deprotonation of this new oxygen to the hydroxyl and of the N3 nitrogen to the neutral species in workup results in the desired product. Reaction of this nucleobase precursor with difluoro-dinitro-benzene resulted in the production of a number of new compounds but not the desired nucleophilic aromatic substation that was seen with the nucleoside, perhaps a result of additional competing nucleophilic sites on the nucleobase.
To begin a protecting group strategy to direct the reaction of the hydroxyl cytosine and difluoro-dinitro-benzene, the N4 nitrogen of 5-hydroxycytosine was tested for protection as a phthalimide (figure 3-0-16). The starting material was reacted with phthalic anhydride in hydrochloric acid and then potassium bicarbonate to neutralize. Acidification of the phthalic anhydride increases its electrophilicity. Nitrogen of the primary amine at the N4 of the cytosine attacks the carbonyl carbon of the protonated anhydride. Reformation of the double bond expels the anhydride oxygen which can deprotonated the nitrogen cation. Attack at the other carbonyl carbon forms the phthalimide and water is expelled upon reformation of the carbon oxygen double bond. Although a purple solid was generated under these conditions, it was not the desired phthalimide protected 5-hydroxycytosine. Following purification starting material was the only isolated compound.

![Figure 3-0-16: Phthalimide protection of hydroxy-cytosine. Protection of the exocyclic amine of hydroxy cytosine so the N1 nitrogen could be protected to prevented unwanted side reactions in tricyclic cytosine nucleobase formation.](image)

Additional attempts at a more successful reduction of the dinitro nucleoside were made using sodium borohydride and sulfur in THF (figure 3-0-17). Sulfurated sodium borohydride has been used to reduce aromatic dinitro compounds to the corresponding amines. Sodium borohydride reacts with sulfur to generate the sulfurated sodium borohydride and this then reacts with the nitro starting material. The hydride shift is the first step of the mechanism and the oxygen can attack the boron to form the borate.
The sulfur is also believed to be implicated in the reduction mechanism but the mechanistic role of all elements is not fully understood.\textsuperscript{120} Initially under these conditions no reaction was observed so additional sulfuratated borohydride was generated and added to the reaction but to no avail. Sulfur flowers were substituted as the sulfur source in the generation of the borohydride, but this still did not result in reduction of the nucleoside even when subjected to 220 °C.

![Figure 3-0-17](image)

**Figure 3-0-17:** Reduction with sulfuratated borohydride. Sulfuratated borohydride was used to reduce the nitro groups to amines en route to alkyl amine functionality.

The same starting material as that used to form the dinitro analog was used in the synthesis of the tetrafluoro analog through the same chemistry (figure 3-0-18). Hexafluoro-benzene was combined with the starting material in DMSO with potassium carbonate.\textsuperscript{121} The basic environment increases the nucleophilicity of the cytosine hydroxyl and amine functionalities and they undergo nucleophilic aromatic substitution at the hexafluorobenzene to give the desired tetrafluoro tricyclic cytosine product.

![Figure 3-0-18](image)

**Figure 3-0-18:** Tetrafluoro-\textsuperscript{1}C\textsuperscript{9} synthesis. Tetrafluoro tricyclic cytosine analog that was synthesized in one step from hydroxy cytidine by reaction with hexafluoro-benzene.
Other Oxygen Tricyclic Cytidine Analogs Experimental:

7,8-dinitro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside.

2′deoxycytidine (150 mg, 0.617 mmol) was combined with 1,2-difluoro-4,5-dinitrobenzene (126 mg, 0.617 mmol) under N₂. Triethylamine (350 μL, 2.5 mmol) was added and the reactants dissolved in dry DMF (7.5 mL) and heated to 65 degrees. 18 hours later the reaction was evaporated and purified via flash silica gel chromatography, and triturated with methanol to give product (60 mg, 0.147 mmol) in 24% yield. ¹HNMR (CD₃OD) δ 7.78 (s, 1H), 7.40 (s, 1H), 7.28 (s, 1H), 6.20 (t, J = 6.5 Hz, 1H), 4.38 (dt, J=6.4, 3.6 Hz, 1H), 3.93 (d, J=3.2 Hz, 1H), 3.87 – 3.64 (m, 2H), 2.31 (s, 1H), 2.16 (dt, J=13.4, 6.6 Hz, 1H), 0.89 (dd, J=15.3, 8.6 Hz, 3H).
7,8-diamino-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. 7,8-dinitro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside (19 mg, 0.0466 mmol) was combined with 10% palladium on charcoal (1 mg) and dissolved in ethanol (1mL) under N₂. After heating to 70 °C, hydrazine monohydrate (48 μL, 0.982 mmol) was added dropwise and the reaction continued at 70 degrees. After 3 hours the reaction was cooled to room temperature and purified via preparatory thin layer chromatography to give crude product that was used in acetylation reaction.

Hydrogen cytosine-5-sulfate monohydrate. Cytosine (2.08 g, 18 mmol) was dissolved in 1N potassium hydroxide (100 mL). Potassium persulfate (7.3 g, 27 mmol) was added to the reaction and stirred at room temperature. After 20 hours concentrated hydrochloric acid (9 mL) was added to the reaction and product precipitated. Chilled filtration, washing with water and acetone gave product (1.7 g, 8.2 mmol) in 45% yield. 1H NMR ((CD₃)₂SO) δ 8.11 (s, 6H), 7.47 (s, 1H).
5-hydroxycytosine. Hydrogen cytosine-5-sulfate monohydrate (1.5 g, 7.2 mmol) was dissolved in 6 N hydrochloric acid (3.5 mL) and this was stirred at 100 °C. After 15 minutes the reaction was chilled on ice. Filtered product was dissolved in deionized water (30 mL) and brought to pH 7 by addition of potassium hydroxide. Product was filtered, washed with water and acetone to give product (677 mg, 5.3 mmol) in 74% yield. 1H NMR ((CD$_3$)$_2$SO) δ 8.31 (s, 1H), 7.40 (s, 1H), 6.90 (s, 3H).

6,7,8,9-tetrafluoro-1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside.

2’deoxy-5-hydroxycytidine (120 mg, 0.494 mmol) was combined with hexafluoro-benzene (47 μL, 0.494 mmol) in N$_2$ and dissolved in DMSO (6 mL). Potassium carbonate (274 mg, 1.98 mmol) was added to the reaction and it was heated to 50 °C. After 18 hours the reaction was cooled to room temperature and spread on a watch-glass to evaporate. The residue was purified via flash silica gel chromatography to give the product (66 mg, 0.169 mmol) in 34% yield. $^1$HNMR (CD$_3$OD) δ 7.46 (s, 1H), 6.20-6.14 (m, 1H), 4.34 (dt, J=6.3,3.3 Hz, 1H), 3.86 (q, J=3.2 Hz, 1H) 3.78-3.65 (m, 2H), 2.21-2.10 (m, 2H). $^{19}$F NMR (CD$_3$OD) δ -159.95, -167.35, -167.80, -169.79.
Oxygen Tricyclic Cytidine Analogs Elaboration

The nucleosides that were synthesized under these synthetic methods were elaborated upon to apply them to further investigations. Triphosphates are popular with biochemists because it makes the nucleoside a substrate for enzymes. These are useful for experimental investigations of cellular activity that take advantage of the photophysics of the nucleotide as well as using enzymes to produce nucleic acids which contain the new fluorophores.

Figure 3-0-19: Triphosphate synthesis. Ludwig method was used in the synthesis of triphosphates from substituted 1C’ nucleosides for enzymatic incorporation.

The high yielding ribose nucleoside of tricyclic cytosine converted to the triphosphate via the Ludwig method. Phosphorous oxychloride provides the alpha phosphate and tributyl ammonium pyrophosphate is the source of the beta and the gamma (figure 3-0-19). Substitution for the chlorine of phosphorous oxychloride by the 5’ hydroxyl oxygen followed by deprotonation gives the intermediate monophosphate analog. Tributylamine serves as a base to deprotonated the tributylammonium pyrophosphate and it will substitute for chlorine on this analog of monophosphate creating the nucleotide triphosphate. Water substitutes for the final chlorine and deprotonation gives triphosphate that is recognizable by polymerase enzymes. Ion exchange chromatography of the reaction mixture was attempted in triethylammonium acetate buffer and this was not an effective mobile phase. Switching to triethylammonium
bicarbonate buffer was much more effective in eluting the various phosphate versions of the fluorescent nucleotide in high yield. To garner compounds with higher purity, the eluent from ion exchange column of the next reaction mixture was subjected to HPLC purification with triethylammonium bicarbonate buffer and acetonitrile. This process was repeated to make the triphosphate of the deoxyribose version of tricyclic cytosine as well.

Ion exchange chromatography being a potentially automated process, the reaction mixture was used as a demonstration of flash ion exchange chromatography. This demonstration was unsuccessful, and the compound was never recovered. To possibly increase the reactivity of the compounds, proton sponge was added to the reaction as this was shown to be beneficial for other triphosphate synthesize. No evidence of reaction between the nucleoside and either the phosphorous oxychloride or the pyrophosphate was observed in these conditions, and the proton sponge was never used again. Skipping the ion exchange altogether, and instead purifying multiple times by HPLC led to the desired product of the tetrafluoro analog previously described. 7,8-NO2-tC⁰-dRTP to which the tetrafluoro analog was to be paired for incorporation investigations was also prepared this way. The same method resulted in the confirmation through mass spectroscopy of the triphosphate nucleotide of tetC⁰.

Changing to a new HPLC column required a restart in method development for the purification of these compounds, and with concentrations low it is difficult to get reliable phosphorous spectra for all nucleotide products. Mass spectral analysis was turned to as a means to confirm the presence of the desired product, but it was later determined by enzymatic incorporation that compounds which did not have the predicted
molecular ion detected by mass spectroscopy were in fact the triphosphate product. HPLC analysis in combination with proton and phosphorous NMR were used to determine the potential triphosphate products of the other substituted tC analogs.

Analytical HPLC analysis of the reaction provided a means of investigating the reactivity of the nucleosides in these conditions. Reaction with phosphorous oxychloride happens quickly as is evidenced by a large shift in the retention time of a sample of the reaction following reactant addition. Reaction with tributylammonium pyrophosphate is less obvious and appears to be a slower process. Coordination between the analytical column and the preparatory column was difficult because elution profiles were very different, even though the stationary phases were the same.

Figure 3-0-20: Solid phase DNA synthesis preparation. Installation of dimethoxytrityl group at the 5′ and phosphoramidite group at the 3′ position of nucleosides for solid phase DNA synthesis to incorporate these substituted tC analogs.

For solid phase DNA synthesis, a dimethoxytrityl group must be installed in the place of phosphate at the 5′ position of the nucleoside and a phosphoramidite group placed at the 3′ position (figure 3-0-20). Because of the greater reactivity of the 5′ hydroxyl, the dimethoxy trityl group is installed first. The hydroxyl oxygen attacks the tertiary carbocation of the trityl after chlorine has departed in a unimolecular substitution.
mechanism. Deprotonation affords the desired neutral product. In the parent compound synthesis the crude reaction mixture from the ring closure step is protected. Attempting this crude tritylation with the tetC° compound resulted in a large mess that even after purification by preparatory thin layer chromatography could not be resolved. 8-OMe-tC° which had been purified and evaporated from dry solvent showed limited reactivity to the tritylchloride as well, and purification was difficult. Multiple additions of the trityl chloride to the 2’deoxyribose of tC gave evidence of product formation but purification was difficult. The 7-Cl-tC° was the first analog to show complete conversion of starting material under these conditions, but the protecting group was cleaved during purification by column or by plate. Other reactions showed dimethoxytritylation of the ribose but fracturing of the glycosidic bond as well. Adding triethylamine to the mix, to promote reaction, was attempted but showed no increased reactivity of starting material. Triethylamine in combination with DMAP showed an improvement in reaction, but purification issues persisted. After testing several possible sets of reaction conditions, the best reactivity was obtained using DMAP in combination with the starting material and dimethoxytritylchloride in pyridine. Starting materials that were as dry as possible were important to the reaction, as well as keeping reactants as concentrated as possible. Purification via flash silica gel chromatography with dichloromethane and methanol with 1% triethylamine resolves the desired product effectively. Unfortunately the triethylammonium ions that result from this purification strategy are difficult to remove. These ions do not appear to have an effect on the installation of the phosphoramidite group so the dimethoxytritylated compounds produced were carried on as a
triethylammonium salt. Purification via diol chromatography was attempted, but conditions where separation and elution both occur have yet to be determined.

With the dimethoxytrityl group installed, it was time to work on the phosphoramidites. The 3’ hydroxyl oxygen attacks the phosphorus of the 2-cyanoethyl-diisopropylamino-chlorophosphoramidite substituting for chlorine to give the desired product. Early reaction between the 5’ protected dinitro analog and phosphoramidite precursor with diisopropylamine in THF went quickly at room temperature but multiple products were difficult to distinguish. Similar results were seen months later with the tetC. Over a year and a half later, the solvent was switched from THF to dichloromethane and this had positive effects on yield. Lowering the temperature of the reaction increased the selectivity. Diisopropyl ammonium tetrazolide as an additive to promote the reaction did lead to appearance of the desired product, but much decomposition occurred upon any attempts to purify the reaction mixture. It was ultimately found that Hunigs base gave the greatest yield of isolable product from the reaction. Purification via flash silica gel chromatography with dichloromethane and methanol with 1% triethylamine is sometimes effective to separate product but creates similar issues to those described above. Alternatively, purification via a diol stationary phase with a gradient from hexanes to dichloromethane can give pure product. Unfortunately, the yield from these two steps is not large enough to produce compounds in amounts suitable for solid phase DNA synthesis even when starting from 150mg of the pure nucleoside. The 8-OMe-tC analog, which can be generated in great amounts, has
been subjected to these reaction conditions and after application of the optimized purification conditions, may be suitable for this means of nucleic acid production.

Another elaboration that was considered was the trifluoromethylation of the oxygen tricyclic cytosine. This electron withdrawing group could provide some potentially interesting fluorescence properties like those seen in the following chapter. Moreover, electron withdrawing groups tend to minimize photobleaching by raising the oxidation potential. To check the utility of trifluoromethylation, a non tC fluorescent nucleoside was trifluoromethylated firsts, pyrrolo-dC (figure 3-0-21). This biphasic reaction helps to control the reagent addition to the reaction. 126 Tert-butoxide becomes the butoxy radical with the assistance of zinc. This radical abstracts an electron from the sulfinate anion generating the sulfinate radical which becomes the trifluoromethyl radical upon loss of sulfur dioxide. Trifluoromethyl radical reacts with the double bond of the

Figure 3-0-21: Trifluoromethylation of fluorescent nucleotides. Trifluoromethyl group substitution was made to determine effects of this functionality on photophysical properties. Conditions were tested on Pyrrolo-dC prior to reaction with the tC0 compounds.
nucleotide and hydroxide radical from tert-butoxide abstracts a hydrogen to give the product and radical as neutral species and another butoxy radical for the cycle.\textsuperscript{126} To ensure the solubility of the starting material in the organic phase, the hydroxyls were acetylated. This reaction with pyrrollo-dC and with tC\textsuperscript{0} can be tricky because of the potential to acetylate the amine of the chromophore which is evidenced by a loss of fluorescence of the compound. Success in the trifluoromethylation of the non-tricyclic compound was encouraging, but selectivity may be an issue with the trifluoromethylation of the tC compounds.
Oxygen Tricyclic Cytidine Analogs Elaboration Experimental:

5'-triphosphate-tC-β-D-ribonucleoside. tC-β-D-ribo nucleoside (22 mg, 0.065 mmol) was dissolved in dry trimethyl phosphate (1 mL) and cooled to -6 °C.

Phosphorous oxychloride (6.7 μL, 0.071 mmol) in trimethyl phosphate (200 μL) was added dropwise to the reaction stirred at -25 °C. After 3 hours tributyl ammonium pyrophosphate (251 mg, 0.455 mmol) in dry dimethyl formamide (0.5 mL) was added to the reaction followed by 7 drops of tributylamine and stirred at -23 °C 2.5 hours then at room temperature 1 hr. The reaction was poured into 100 mL of 0.1 M triethyl ammonium bicarbonate buffer and evaporated. Purification via a 6cm column of Sephadex A-25 ion exchange resin that had been swollen with 1M sodium hydroxide and washed with millipure water for 2 hours. The crude residue was diluted with 140 mL deionized water and loaded on to the column. 300 mL of water were run through the column followed by 30 mL each of 20, 40, 60, 80 and 100% triethyl ammonium bicarbonate buffer and sometimes HPLC in a gradient of the buffer to acetonitrile to give the product as the triethyl ammonium salt in less than 15% yield.
2'-deoxy-5'-triphosphate-tC-β-D-ribonucleoside. 2’-deoxy-tC-β-D-ribonucleoside (27 mg, 0.08 mmol) was stirred in trimethyl phosphate (2 mL) over molecular sieves. Heated to 33 °C to dissolve and then cooled to 0 °C. 1.78 M phosphorous oxychloride in trimethyl phosphate (100 μL, 0.056 mmol) was added dropwise to the reaction and stirred at 0 °C for 2 hours. After 2 hours at room temperature, tributyl ammonium pyrophosphate (306 mg, 0.56 mmol) in dry dimethyl formamide (0.56 mL) was added to the reaction followed by several drops of tributylamine and stirred at 0 °C 1 hour then at room temperature. Following 1 hour the reaction was poured into 100 mL of 0.1 M triethyl ammonium bicarbonate buffer and evaporated. The crude residue was diluted with 100 mL deionized water and loaded on to the column. 100 mL of water were run through the column followed by 30 mL each of 20, 40, 60, 80 and 60 mL of 100% 1M triethylammonium bicarbonate buffer. NMR of eluent samples identified which fractions contained compounds of interest from this ion exchange. Samples with proton or phosphorous peaks that indicated presence of desired product were diluted with water (1 mL) and injected onto the HPLC and run (CH3CN in 0.1M TEAB) to give the product as the triethylammonium salt in less than 14% yield.
6,7,8,9-tetrafluoro-1,3-diaza-2-oxophenoxazine-2’-deoxy-5’triphosphate-β-D-ribofuranoside. 6,7,8,9-tetrafluoro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside (30 mg, 0.077 mmol) dry from desiccation was dissolved in dry trimethyl phosphate (1 mL) under N₂, heated to 40 °C to dissolve and then cooled to 0 °C. Dry phosphorous oxychloride (16 μL, 0.171 mmol) was added to the reaction and stirred at 0 °C for 1 hour. After 4 hours at room temperature, tributyl ammonium pyrophosphate (298 mg, 0.54 mmol) in dry dimethyl formamide (0.7 mL) was added to the reaction followed by 7 drops of tributylamine and stirred at room temperature. The reaction was poured into 100 mL of 0.1 M triethyl ammonium bicarbonate buffer and evaporated. The crude residue was diluted with water filtered and injected onto the HPLC and run (CH₃CN in 0.1M TEAB) to give the product (3.3 mg, 0.0052 mmol) in 7 percent yield.

7,8-dinitro-1,3-diaza-2-oxophenoxazine-2’-deoxy-5’triphosphate-β-D-ribofuranoside. 7,8-dinitro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside (15mg, 0.0368 mmol) was dissolved in dry trimethyl phosphate (0.55 mL) under N₂, heated to 47 °C to dissolve and then cooled to 0 °C. Dry phosphorous oxychloride (7.6
μL, 0.0815 mmol) was added to the reaction and stirred at 0 °C for 1 hour. After 5 hours at room temperature, tributyl ammonium pyrophosphate (142 mg, 0.259 mmol) in dry dimethyl formamide (0.25 mL) was added to the reaction followed by 4 drops of tributylamine and stirred at room temperature. Following two hours, the reaction was poured into 50 mL of 0.1 M triethyl ammonium bicarbonate buffer and evaporated. The crude residue was diluted with water filtered and injected onto the HPLC and run (CH3CN in 0.1M TEAB) to give the product (0.2 mg, 0.000309 mmol) in 0.8 percent yield.

8-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-5’triphosphate-β-D-ribofuranoside. 8-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. (50 mg, 0.142 mmol) was dissolved in dry trimethyl phosphate (0.55 mL) under N₂, then cooled to 0 °C. Dry 1M phosphorous oxychloride in trimethyl phosphate (156 μL, 0.156 mmol) was added dropwise to the reaction and stirred at 0 °C for 15 minutes then at room temperature 2 hours. Recooled to 0 °C, additional dry 1M phosphorous oxychloride in trimethyl phosphate (156 μL, 0.156 mmol) was added dropwise to the reaction and stirred at 0 °C. After 30 minutes it was warmed to room temperature and stirred 2 hours at room temperature. Recooled to 0 °C, tributyl ammonium pyrophosphate (951 mg, 1.73 mmol) in dry dimethyl formamide (1 mL) was added to the reaction dropwise followed by 14
drops of tributylamine and stirred at 0 °C for 35 minutes then warmed to room temperature. The reaction was evaporated, diluted with 5 mL of 0.1 M triethyl ammonium bicarbonate buffer filtered and injected onto the HPLC and run (CH3CN in 0.1M TEAB) to give the product (1.9 mg, 0.00321 mmol) in 2 percent yield.

1,3-diaza-2-oxophenoxazine-2'-deoxy-5'triphosphate-β-D-ribofuranoside was synthesized for analytical method development. Samples were diluted with water and then injected (20 μL) onto the HPLC and run (CH3CN in 0.1M TEAB). 1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside (28 mg, 0.0898 mmol) was dried overnight in vacuo then dissolved in dry trimethyl phosphate (0.5 mL) under N2 (Sample A). Reaction was cooled to 0 °C. Dry 1M phosphorous oxychloride in trimethyl phosphate (98 μL, 0.098 mmol) was added dropwise to the reaction and stirred at 0 °C for 20 minutes (Sample B) then at room temperature 1 hour (Sample C). Chilled tributyl ammonium pyrophosphate (246 mg, 0.45 mmol) in dry dimethyl formamide (0.3 mL) was added to the reaction dropwise followed by 8 drops of tributylamine and stirred for 1 hour (Sample D). After an additional hour (Sample E) and at 3 total hours (Sample F).

General Methods for the synthesis of 1,3-diaza-2-oxophenoxazine-2'-deoxy-5'triphosphate-β-D-ribofuranosides.

Method I: 1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranosides (50 mg, 0.142 mmol) that have been dried overnight in vacuo are dissolved in dry
trimethylphosphate (0.4 mL) under N\textsubscript{2}. Heated and sonicated to dissolve, the reaction is
then cooled to 0 °C. Chilled 1M phosphorous oxychloride solution in dry
trimethylphosphate that has been dried over 3 angstrom molecular sieves (156 \mu L, 0.156
mmol) is added dropwise to the reaction and stirred at 0 °C. After 30 minutes the reaction
is warmed to room temperature and stirred for one hour then returned to 0 °C. A 0.4 M
solution of tributylammonium pyrophosphate in dry dimethylformamide that has been
dried over 3 angstrom molecular sieves (1.8 mL, 0.72 mmol) was added dropwise to the
reaction followed by several drops of tributylamine. After stirring at 0 °C for 30 minutes,
the reaction is warmed to room temperature. Following 3 hours at room temperature the
reaction is poured into 0.1 M triethyl ammonium bicarbonate buffer (50 mL) and
evaporated. Dilution of the crude residue to 10 mL with 0.1M triethyl ammonium
bicarbonate buffer, syringe filtration and preparatory HPLC purification (CH3CN in
0.1M TEAB) gives the product (2.6 mg, 0.00439 mmol) in 3% yield.

7-methoxy-1,3-diaza-2-oxophenoxazine-2’-deoxy-5’triphosphate-\beta-D-
ribofuranoside. Synthesized via method I from 7-methoxy-1,3-diaza-2-oxophenoxazine-
2’-deoxy-\beta-D-ribofuranoside. Less than 5% yield

1,3-diaza-benzo(h)2-oxophenoxazine-2’-deoxy-5’triphosphate-β-D-ribofuranoside. Synthesized via method I from 1,3-diaza-benzo(h)2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. Less than 1% yield.

7-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-5’triphosphate-β-D-ribofuranoside. Synthesized via method I from 7-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. Less than 3% yield.

General Methods for the synthesis of 1,3-diaza-2-oxophenoxazine-2’-deoxy-5’-dimethoxytrityl-β-D-ribofuranosides.
Method J: 1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranosides (100 mg, 0.272 mmol) that have been dried overnight in vacuo with dimethylaminopyridine (1.7 mg, 0.0139 mmol) and dimethoxytrityl chloride (115.6 mg, 0.322 mmol) were dissolved in dry pyridine (0.4 mL) under N₂ and stirred at room temperature until reaction stopped proceeding as apparent by thin layer chromatography. Purification via flash silica gel chromatography (CH₃OH in CH₂Cl₂ with 1% TEA) gives the semicrude product.

**8-chloro-1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside.** Synthesized via method J from 8-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 21% yield.

![Chemical structure](image)

**7-chloro-1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside.** Synthesized via method J from 7-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 10% yield.
8-methoxy-1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside. Synthesized via method J from 8-methoxy-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 46% yield.

1,3-diaza-benzo(h)2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside. Synthesized via method J from 1,3-diaza-benzo(h)2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 15% yield.
8-methoxy-tC-5’-dimethoxytrityl-2’-deoxy-β-D-ribonucleoside. Synthesized via method J from 8-methoxy-tC-2’-deoxy-β-D-ribonucleoside in less than 61% yield.

General Methods for the synthesis of 1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-3’ phosphoramidite-β-D-ribofuranosides.

Method J: 1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranosides (29.6 mg, 0.0455 mmol) that have been dried overnight in vacuo with dimethylaminopyridine (0.3 mg, 0.00228 mmol) were dissolved in dry dichloromethane (0.3 mL) under N₂ and cooled to 0 °C. Ethyl-diisopropylamine (10 µL, 0.0605 mmol) and 2-cyanoethyl-diisopropylamino-chlorophosphoramidite (11 µL, 0.0455 mmol) were added dropwise. After reaction appeared complete by thin layer chromatography (5 minutes) reaction was diluted with methanol and evaporated. Purification via flash silica gel chromatography (CH₃OH in CH₂Cl₂ with 1% TEA) gives the crude product.
8-chloro-1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-3’-phosphoramidite-2’-deoxy-β-D-ribofuranoside. Synthesized via method K from 8-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 12% yield.

8-methoxy-1,3-diaza-2-oxophenoxazine-5’-dimethoxytrityl-3’-phosphoramidite-2’-deoxy-β-D-ribofuranoside. Synthesized via method K from 8-methoxy-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside in less than 32% yield.
1,3-diaza-benzo(h)2-oxophenoxazine-5’-dimethoxytrityl-3’-phosphoramidite-2’-deoxy-β-D-ribofuranoside. Synthesized via method J from 1,3-diaza-benzo(h)2-oxophenoxazine-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside in less than 7% yield.

8-methoxy-tC-5’-dimethoxytrityl-3’-phosphoramidite-2’-deoxy-β-D-ribofuranoside. Synthesized via method J from 8-methoxy-tC-5’-dimethoxytrityl-2’-deoxy-β-D-ribofuranoside in less than 10% yield.
**3’,5’dicetyl-Pyrrolo-dC.** Pyrrolo-dC (50 mg, 0.188 mmol) was placed dissolved in dry pyridine (0.6 mL) under N₂. Acetic anhydride (60 μL, 0.636 mmol) was added and the reaction stirred at room temperature until completed as seen by thin layer chromatography. Purification via flash silica gel chromatography resulted in product. (13 mg, 0.372 mmol) in 20% yield. 

\[ ^1H \text{ NMR (CDCl}_3) \delta 12.62 (s, 1H), 8.12 (s, 1H), 6.44 (dd, J=7.7, 5.6 Hz, 1H), 5.85 (q, J=1.2 Hz, 1H), 5.24 (dt, J=6.6, 2.4 Hz, 1H), 4.48 – 4.33 (m, 3H), 2.88 (ddd, J=14.4, 5.6, 2.3 Hz, 1H), 2.49 (d, J = 1.2 Hz, 3H), 2.17 – 2.11 (m, 1H), 2.10 (s, 3H), 2.07 (s, 3H). \]

**3’,5’dicetyl-7-trifluoromethyl-Pyrrolo-dC.** Zinc trifluoromethanesulfinate (9.3, 0.0596 mmol) was dissolved in dichloromethane (0.25 mL) under N₂. 3’,5’dicetyl-Pyrrolo-dC (6.6 mg, 0.019 mmol) was dissolved in dichloromethane and added to the reaction followed by deionized water (0.2 mL). An additional water (0.2 mL) and dichloromethane (0.75 mL) were added to the reaction and cooled to 0 °C. 70% tert-butoxide (13 μL, 0.108 mmol) was added dropwise and the reaction stirred. Following
completion of reaction via thin layer chromatography, the reaction was diluted with water and dichlormethane. The aqueous layers were washed with dichloromethane 2 times and the combined organics were dried and evaporated. Purification via flash silica gel chromatography resulted in product (2.2 mg, 0.00527 mmol) 28% yield. $^1$H NMR (CDCl$_3$) δ 8.37 (s, 1H), 6.44 (dd, J=7.8, 5.6 Hz, 1H), 5.26 (d, J=6.4 Hz, 1H), 4.42 (q, J=4.9, 3.9 Hz, 3H), 2.91 (ddd, J=14.7, 5.7, 2.0 Hz, 1H), 2.66-2.61 (s, 3H), 2.19 – 2.13 (m, 1H), 2.12 (d, J=0.8 Hz, 3H), 2.08 (d, J=0.8 Hz, 3H). $^{19}$F (55.40).
Chapter 4 Photophysics of Tricyclic Cytosine Analogs

Introduction

Analogs were investigated for the changes in photophysical behavior from parent compounds tC and tC° imparted by the substitutions that were appended to the fluorophore skeleton in the course of the synthetic work (Chapters 2-3, figure 4-0-1). As discussed in the introduction to this dissertation, there is a complicated relationship between a molecule’s structure and its ability to interact with light. This relationship is so complicated that it is difficult to make predictions on what substitutions will lead to a desirable effect, and therefore the ability to use rational design is limited when planning synthesis for desired properties. Through the series of closely related compounds synthesized in this work it was possible to look for patterns in how the substituent

Figure 4-0-1: Analogs investigated for relationships between structure and photophysical properties
character influenced quantum efficiency. A goal of this work is to use these substituent effects in future rounds of iterative design. Photophysical characterizations were made in both an aqueous and an organic environment. Mixtures of solvents were utilized in order to determine the sensitivity of these photophysical properties to the environment of study.

Although wavelength of maximum absorbance varied only slightly through the series of compounds, the absorptivity displayed some interesting variance and solvent sensitivity. The absorptivity contributes to the brightness of an analog, and therefore it is desirable to have the greatest absorptivity. Molecules that exhibit an increase in fluorescence brightness upon formation of higher order structures, such as single strands double strands of nucleic acids or as part of an enzyme, are desirable experimental tools. A turn on signal is easy to distinguish, and eliminates much background signal. The degree to which a compound interacts with the light is an intrinsic property and therefore the experiments are limited by the probes and labels that are available.

Emission wavelength and the dependent Stokes shift of some of the analogs showed variation from the parent compound. Quantum yield varies between the molecules investigated, as well as the brightness, which is the mathematical product of the varying absorptivities and quantum yields. These variances when examined collectively do not correlate with the characteristic Hammett values or the differences in

Figure 4-0-2 Analog peripherally investigated. 7,8-NO2-tC-dRTP was not included in the free nucleoside study but absorbance and emission data were collected in buffer.
the Hammett value from that of the parent compounds. However, when looking at individual positions of substitution on the chromophore, some correlation to Hammett constant occurs. Conflicting results on the solvent sensitivity of the substituted tricyclic cytosine analogs were obtained from a Reichardts dye comparison study and examination of the photophysical behavior of the compounds in dioxane versus buffer. This lack of correlation is not necessarily surprising, because the polarity of an environment is determined by multiple factors. Polarity comes from the dielectric relaxation of the solvent, or how long the dielectric constant takes to change in response to a stimulus. Electronic polarizability affects polarity, as the tendency of the charge distribution of the media to be distorted. Solvent quadrupole reorientation also determines environment through the configuration of the dipoles.\textsuperscript{38} Explanations of these substituent effects are difficult, but data can be compared to the results from similar investigations.

Additionally, the dinitro analog triphosphate was investigated in buffer, for its absorbance and fluorescence properties (figure 4-0-2). Comparisons between this analog are aided by the characterization of a single nitro substitution on the tC framework that is found in the literature.\textsuperscript{61}
Tricyclic cytosine analogs are seen to be stabilizing to the duplex, and generally the greatest stability is seen to result in the lowest quantum yield, likely because of excited state relaxation through a charge transfer mechanism.\textsuperscript{31} Dipole moment differences between the excited and the ground state can lead to an influence on emission wavelength of the compound by the environment.\textsuperscript{6} Time resolved measurements would provide information on the dynamics of the excited state and the decay pathways of the fluorophore, but this investigation focused on the steady state behavior of the compounds.

In a Reichardts dye comparison experiment, the analogs are placed in various mixtures of a polar and nonpolar solvent and the change in Stokes shift is compared to the absorbance wavelength of the aforementioned dye (figure 4-0-3). The dye has a change in absorbance wavelength depending on solvent polarity because of polarity differences in the ground and excited states. Ground state zwitterion dye molecules become neutral upon excitation.\textsuperscript{127} Therefore nonpolar solvents result in a smaller HOMO-LUMO energy gap than polar solvents, and the wavelength of absorbance is affected accordingly. From this experiment it was shown that the most sensitive to environment polarity was the 7-OMe-tC analog followed closely by tetC\textsuperscript{o}. Contrarily, the greatest difference in Stokes shift between the two solvents for a compound was found with the exceptionally bright 8-Cl-tC\textsuperscript{o} with 43 nm difference.
Absorption of analogs

Once the substituted analogs had been synthesized through the means discussed above, it was possible to investigate their interactions with light from an absorbance standpoint. The unsubstituted oxygen tricyclic cytosine analog tC⁰ was observed to have an average absorbance wavelength of 356 nm in buffer and 357 nm in dioxane. This indicates there was little difference in the polarity of the ground versus the excited state as the energy gap was unchanged between these solvents. This is close to the literature value reported for this compound in buffer that was determined to be the result of a single absorption transition moment.⁴² Investigations of this compound also revealed that there were not strong effects of pH, salt or temperature, like the lack of change observed in different environments in this investigation. There is a large absorbance centering around 220 nm as well as one the one discussed above. The 356nm absorbance is a desirable wavelength for probe absorbance because it is removed from the absorbance of the natural nucleosides and avoids potentially complicating coupling effects.⁶⁶

An aqueous absorptivity for tC⁰ of 6800 M⁻¹ cm⁻¹ is increased to 7400 M⁻¹ cm⁻¹ in dioxane. A value of 6800 M⁻¹ cm⁻¹ in buffer is lower than that which is reported in the literature, and is not as significantly better than the sulfur containing homologue. Differences in measurements are not uncommon, and therefore tC was utilized as a second standard in all measurements made.¹²⁸ This difference in absorptivity depending on environment is not surprising given that there are slight changes in the fluorescence intensity and lifetime reported for the molecule upon incorporation in nucleic acid oligomers. Although there is some slight fine structure in the spectra collected, there is
not the vibrational mode display discussed in the literature for this molecule. The following Table 5 is a table of the absorptive properties of the analogs investigated.

Table 5: Absorbance of analogs are presented with wavelengths in nm and absorptivities of M⁻¹ cm⁻¹.

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A slightly larger environmental difference was seen for tC in extinction coefficient than that observed for tC, and unlike tC, this compound is more absorbing in dioxane. The change from a bent to a planar conformation upon absorption by the tC
compound could explain why it is more sensitive, as this has also been used to explain this compound’s larger Stokes shift which is discussed later. The smaller change in absorption coefficient due to environment agrees with the literature decreasing upon base stacking in oligonucleotides. The values acquired for this parent compound agree more closely with the literature value of 4500 M\(^{-1}\) cm\(^{-1}\) in buffer. An absorbance wavelength shift reported in the literature for the analogs upon stacking is not mimicked in switching sample solvent, in dioxane the absorbance wavelength went 8 nm to the blue, whereas upon stacking the absorbance maximum was shown to shift 25 nm to the red.

The absorptivities of the members of this tricyclic family are smaller than some of the other analogs that are available, but they are better upon evaluation by the other criteria discussed. pH-sensitive analogs that are also fluorine NMR probes have large absorptivities in methanol, from 18000-38000 M\(^{-1}\) cm\(^{-1}\). Alkynyl derivatives of uridine have absorptivities ranging from 4100 to 19450 M\(^{-1}\) cm\(^{-1}\). Comparative extinction coefficients ranging from 2420-15000 M\(^{-1}\) cm\(^{-1}\) are seen by Pyrrolo-C analogs. Being able to absorb more photons is beneficial because it increases the energy that could potentially be emitted as photons. Generally increasing the size of the chromophore can increase the absorptivity.
By this rationale, tetC⁰ would be expected to have a greater absorptivity, but this was not observed to be the case with the system under study. The absorption cross section is proportional to the extinction coefficient, but this larger aromatic system did not increase. A great decrease of 1533 M⁻¹ cm⁻¹ in absorptivity was seen upon the addition of the fourth ring to the skeleton in buffer and there was a less but still measurable 704 M⁻¹ cm⁻¹ decrease in absorptivity from tC⁰ to tetC⁰ in dioxane. The additional conjugation provided in this analog is expected to lower the HOMO and the LUMO energies and result in the absorbance having a red shift. This decrease in energy does not explain the decreased population of the excited state for this compound. A sizable 1413 M⁻¹ cm⁻¹ absorptivity difference between the environments tested suggests that this may be an environment issue. Non-degenerate interaction causes a decrease in the intensity of absorption of nucleic acids in DNA and perhaps hydrophobic interactions create a similar interaction with these extended compounds in aqueous solvent.

The addition of another aromatic ring to the chromophore did not significantly alter the absorbance wavelength of this system, or the sensitivity of this property to the environment. FBAs with extended aromatic surfaces were shown to have absorbance...
bands shift, making tetC° unique. TetC° had an average absorbance wavelength in buffer of 361 nm and in dioxane of 362 nm. Red shifting upon stacking of the tricyclic cytosine analog was not simulated by the nonpolar solvent in the study, and could potentially be extended by the tetracyclic version. Further red shifting than the sulfur parent compound due to enhanced stacking by tetC° might just land an absorbance wavelength in a desirable FRET pairing region. There would also be an expectation of an increase in the fine structure of the tetC° compound absorbance spectra, because of the different conformations that are possible and the different vibrational levels for each of the energy states. Shouldering in the absorbance peak indicates the presence of several different transitions. As predicted, there is a greater degree of structure in the absorbance for this compound compared with the other substitutions studied.

Greater absorptivity was only seen when a chlorine substituent was present, and only in dioxane was this increase observed. In dioxane, 1094 M⁻¹ cm⁻¹ greater absorptivity was had by the 7-Cl-tC° in comparison to the parent compound and even greater still the 8-Cl-tC° analog increased 1275 M⁻¹ cm⁻¹. An absorptivity increase has been seen in the substitution of aniline by chlorine as well. In this work the position of the chlorine substituent had only slight influence on the absorptivity as well. In buffer these analogs had a decreased absorptivity of 1175 M⁻¹ cm⁻¹ and 67 M⁻¹ cm⁻¹ respectively. The 2853 M⁻¹ cm⁻¹ difference in absorptivity between solvents for the 8-Cl-tC° analog is the largest that was seen for the analogs in this investigation followed by the 1925 M⁻¹ cm⁻¹ difference for the 7-Cl-tC° compound. Solvent dependency of this
phenomena could possibly be a result of the fact that the dipole moments of compounds can be dependent on the position of the chlorine substituent.

In previous studies, substitutions to the framework (methoxy excluded) have been shown to have a collective decrease in the HOMO and the LUMO energies resulting in a bathochromic shift in absorbance wavelength.\textsuperscript{135} It would be beneficial to have absorbance shift far enough to overlap with the emission of another analog, as this would be the beginnings of a potential Forster resonance energy transfer pair. Significant shifts were not seen for any of the analogs, but the largest of 9 nm comes in dioxane from the 8-Cl-tC\textsuperscript{0} and the 8-F-tC\textsuperscript{0} analogs (figure 4-0-4). This 8-F substitution had the largest influence on the absorptivity as well, decreasing 5008 M\textsuperscript{-1} cm\textsuperscript{-1} in buffer and 4687 M\textsuperscript{-1} cm\textsuperscript{-1} in dioxane compared to tC\textsuperscript{0}. This is opposite of the trend seen with other fluorescent nucleosides which had absorptivity increase upon the fluorine substitution.\textsuperscript{136} It has been shown that the resonance effects of a substituent are more important than their inductive influence, and that is why the substituents can be seen to have an energy lowering effect in terms of the HOMO-LUMO transition.\textsuperscript{135} The 7-Cl-tC\textsuperscript{0} analog which has a different position on the ring has a shift of only 6 nm compared to the parent absorbance maximum. This is the same degree of shifting that is seen for 8-Cl-tC\textsuperscript{0} in buffer. Aqueous environments decrease the shifting of 8-F-tC\textsuperscript{0} as well to only 5 nm and 7-Cl-tC\textsuperscript{0} is shifted less than the variance in the measurements.
Aqueous environments were seen to increase the shifting in absorbance maximum for the methoxy substituted compounds. The 7-OMe-tC and 8-OMe-tC shift 5 and 6 nm respectively in buffer while falling underneath the threshold of experimental variance with the degree of shifting observed in dioxane. Shifts were not seen to be in the bathochromic direction as has been seen by ether substitution of other aromatic compounds. However going from buffer to dioxane does result in blue shifts for these compounds. The hydrogen bonding effects of this substituent group are possibly more apparent in a polar solvent. Other shifts that were less than the variance seen in the measurements were those of 7 and 8-OMe-tC (figure 4-0-5). They are not observably different from the absorbance maximum of tC at 369 nm in dioxane and 377 nm in buffer. However of all of the methoxy substituents on either framework, which join tC in having an average red shift in buffer versus dioxane, the sulfur versions show a stronger shift. This greater difference in absorbance based on environment is interesting for the tricyclic cytosine analog because it has been found to be more environmentally insensitive than other fluorescent probes and labels. A difference in absorptivity is seen for tricyclic cytosine between these two environments as well with 4300 M$^{-1}$ cm$^{-1}$ in dioxane.
becoming 4700 M\(^{-1}\) cm\(^{-1}\) in buffer. The opposite trend is observed for 7 and 8-OMe-tC which are both lower in absorptivity than the parent compound. A decrease of 919 M\(^{-1}\) cm\(^{-1}\) for the 8-OMe-tC in buffer from the absorptivity of the parent compound is tempered to only 477 M\(^{-1}\) cm\(^{-1}\) in dioxane. A difference of only 79 M\(^{-1}\) cm\(^{-1}\) in the absorptivity of 8-OMe-tC between the environments is interesting. The 7-OMe-tC behaves similarly although to a greater extent, having absorptivity decrease 2696 M\(^{-1}\) cm\(^{-1}\) in buffer and only 2144 M\(^{-1}\) cm\(^{-1}\) in dioxane.

The opposite trend is seen for this same electronic group when on the tC\(^o\) framework. Absorptivities decreased 4668 M\(^{-1}\) cm\(^{-1}\) in dioxane for 7-OMe-tC\(^o\) in comparison to unsubstituted tC\(^o\) 3796 M\(^{-1}\) cm\(^{-1}\) in buffer. This was the only oxygen containing analog observed to have a stronger interaction with light when in an aqueous environment. 8-OMe-tC\(^o\) had a less solvent dependent 2594 M\(^{-1}\) cm\(^{-1}\) decrease in absorptivity in dioxane, close to the 2791 M\(^{-1}\) cm\(^{-1}\) decrease in buffer.

The addition of a nitro substituent has been shown to be strongly shifting in previous work.\(^6^2\) For the dinitro compound studied, there was a shift observed in the absorbance maxima to the blue (figure 4-0-2). This is another unexpected occurrence, as generally this functionality has been observed to shift absorbance in the opposite direction.\(^6^2\) When there is a dinitro substitution a maximum absorption wavelength of 280 nm was observed. The absorptivity of this compound was found to be much higher than the other analogs in buffer as the triphosphate 16000 M\(^{-1}\) cm\(^{-1}\).

Absorbance wavelength was shown to have no correlation to any Hammett constant in either position of substitution (figure 4-0-7). Absorptivity on the other hand
has some correlation (figure 4-0-8). In either solvent system, the absorptivity is much more strongly correlated to the substitution at the 7 position. This placement on the analog is in the position that is para to the nitrogen. The Hammett constant that gave the best coefficient of determination was the + Hammett constant. These constants attempt to account for both resonance and inductive effects of the substituent. The correlation is slightly less in buffer, which is possible due to the greater influence of hydrogen bonding in this environment.

There is less influence on the absorption than the emission by the polarity of the solvent, because the absorption is assumed to be instantaneous and therefore only subject to the average solvent shell around the chromophore. Polar fluorophores are likely to have enlarged dipoles in the excited state which increases the charge transfer character and leads to a greater dependence of wavelengths on the polarity of the solvent. Hybridization induced tautomerization could also change the electronic nature and thus the photophysics of the chromophore. The absorption also occurs too quickly to allow for motion of the fluorophore or the solvent. Not only do the different water dioxane mixtures have differing polarity, but they have different hydrogen bonding capabilities which complicates the experiments observed.
Figure 406: Absorbance correlation between substituent Hammett constant and analog absorbance wavelength. No linear relationship is seen with substitution at either position of the tricyclic framework. Change refers to the difference in photophysical property as compared to the unsubstituted compound.
Figure 4-0-7: Absorbance correlation between substituent Hammett constant and analog absorptivity. No linear relationship is seen with substitution at position 8 of the tricyclic framework but linear relationship seen between substitution at position 7 in either dioxane or buffer. Change refers to the difference in photophysical property as compared to the unsubstituted compound.
Absorbance Experimental:

UV/VIS spectroscopy was performed using a Varian Cary 100 Bio UV-visible spectrophotometer. Absorbance spectra were obtained using a wavelength range of 600-200 nm, an averaging time of 0.100 s with a data interval of 1 nm. The scan rate was 600 nm per minute with the source changing over set to occur at 200 nm to prevent the jump in absorbance from lamp changing occurring in the area of interest. A baseline correction of the solvent being used was made prior to sample collection and the signal band width was 2.0 nm. Samples were taken from a stock 6 mM solution of each of these analogs and diluted to desired concentrations with the solvent for investigation. A sample of known concentration would be measured for absorptivity, and this value used to calculate concentrations to which the absorbance would be less than 0.1. A 1 cm path length was used. 1X PBS buffer was made from dissolution of phosphate buffered saline tablet in millipure water. One tablet in 200 mL of water gives 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. 0.1 M H₂SO₄ was made from dilution of concentrated sulfuric acid with millipure water. Spectroscopic grade dioxane was used. Quinine sulfate standards was made from dissolution of a measure of quinine hemisulfate in DMSO to create a stock 6 mM solution. Average absorbance wavelengths from 6-13 measurements are reported with standard deviations. Average absorptivities from 2-3 measurements are reported with standard deviations to represent the difference between the measurements.
**8-chloro-1,3-diaza-2-oxophenoxazine-2′-deoxy-β-D-ribofuranoside.** Quinine Sulfate diluted to concentrations of 18, 16, 14, 12, 10, 8, 6, and 4 μM or 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-Cl-tC in 1X PBS buffer at concentrations of 14, 12, 10, 8, 6, and 4 μM and in dioxane at concentrations of 12, 10, 8, 6, 4 and 2 μM. Absorbance maxima of 361 ± 2 nm in buffer 366 ± 1 nm in dioxane. Absorptivity of 5619 ± 1016 M⁻¹ cm⁻¹ in buffer 8472 ± 1018 M⁻¹ cm⁻¹ in dioxane.

**tC 2′-deoxy-β-D-ribonucleoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tC in 1X PBS buffer at concentrations of 22, 20, 18, 16, 14, and 12 μM or 22, 21, 20, 18, 16, and 12 μM or 24, 20, 12, 8, and 4 μM and in dioxane at concentrations of 22, 20, 18, 16, 14, and 12 μM or...
22, 20, 16, 12, 8 and 4 μM. Absorbance maxima of 377 ± 2 nm in buffer 369 ± 1 nm in dioxane. Absorptivity of 4678 ± 207 M⁻¹ cm⁻¹ in buffer 4314 ± 113 M⁻¹ cm⁻¹ in dioxane.

**1,3-diaza-2-oxphenoxazine-2'-deoxy-β-D-ribofuranoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tCo in 1X PBS buffer at concentrations of 14, 12, 10, 8, 6, and 4 μM and in dioxane at concentrations of 12, 10, 8, 6, 4, and 2 μM in duplicate. Absorbance maxima of 355 ± 2 nm in buffer 357 ± 4 nm in dioxane. Absorptivity of 6794 ± 181 M⁻¹ cm⁻¹ in buffer 7378 ± 585 M⁻¹ cm⁻¹ in dioxane.

**1,3-diaza-benzo(h)2-oxphenoxazine-2'-deoxy-β-D-ribofuranoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tetC² in 1X PBS buffer at concentrations of 20, 18, 16, 14, 12, and 10 μM or 20, 16, 12, 8, 4, and 2 μM and in dioxane at concentrations of 12, 10, 8, 6, 4, and 2
μM in duplicate. Absorbance maxima of 360 ± 1 nm in buffer 362 ± 2 nm in dioxane.

Absorptivity of 5261 ± 548 M⁻¹ cm⁻¹ in buffer 6674 ± 1257 M⁻¹ cm⁻¹ in dioxane.

7-chloro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-Cl-tC₀ in 1X PBS buffer at concentrations of 12, 10, 8, 6, 4, and 2 μM and in dioxane at concentrations of 10, 8, 6, 4, 2, and 1 μM in duplicate. Absorbance maxima of 357 ± 2 nm in buffer 363 ± 2 nm in dioxane. Absorptivity of 6278 ± 541 M⁻¹ cm⁻¹ in buffer 8653 ± 179 M⁻¹ cm⁻¹ in dioxane.

7-methoxy-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-OMe-tC₀ in 1X PBS buffer at concentrations of 42, 34, 26, 18, 10, and 2 μM or 36, 30, 24, 18, 12, and 6 μM or 25, 20, 15, 10, 5, and 2.5 μM and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 34, 26, 18, 10, and 2 μM.
Absorbance maxima of 360 ± 2 nm in buffer 358 ± 1 nm in dioxane. Absorptivity of 2999 ± 945 M⁻¹ cm⁻¹ in buffer 2709 ± 7 M⁻¹ cm⁻¹ in dioxane.

8-methoxy-1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-OMe-tCº in 1X PBS buffer at concentrations of 24, 20, 16, 12, 8, and 4 μM and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 18, 15, 12, 9, 6, 3 μM or 18, 14, 10, 8, 6, and 3 μM. Absorbance maxima of 361 ± 1 nm in buffer 360 ± 1 nm in dioxane. Absorptivity of 4004 ± 414 M⁻¹ cm⁻¹ in buffer 4783 ± 411 M⁻¹ cm⁻¹ in dioxane.
8-fluoro-1,3-diaza-2-oxophenoxazine-2′-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-F-tC in 1X PBS buffer at concentrations of 80, 70, 60, 50, 40, and 30 μM or 50, 40, 30, 20, 10, and 5 μM and in dioxane at concentrations of 42, 34, 26, 18, 10, and 4 μM or 50, 40, 30, 20, 10, and 5 μM. Absorbance maxima of 360 ± 1 nm in buffer 366 ± 2 nm in dioxane. Absorptivity of 1787 ± 491 M⁻¹ cm⁻¹ in buffer 2690 ± 628 M⁻¹ cm⁻¹ in dioxane.

7-methoxy-tC-2′-deoxy-β-D-ribonucleoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-OMe-tC in 1X PBS buffer and in dioxane at concentrations of 25, 20, 15, 10, 5, or 2.5 μM in
duplicate. Absorbance maxima of 379 ± 2 nm in buffer 372 ± 3 nm in dioxane.

Absorptivity of 1982 ± 351 M$^{-1}$ cm$^{-1}$ in buffer 2170 ± 180 M$^{-1}$ cm$^{-1}$ in dioxane.

8-methoxy-tC-2'-deoxy-β-D-ribonucleoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H$_2$SO$_4$ were used in comparison with 8-OMe-tC in 1X PBS buffer at concentrations of 24, 20, 16, 12, 8, and 4 μM or 30, 23, 15, 11, 7, and 3 μM in duplicate and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 24, 20, 16, 12, 8, and 4 μM. Absorbance maxima of 379 ± 4 nm in buffer 371 ± 1 nm in dioxane. Absorptivity of 3758 ± 261 M$^{-1}$ cm$^{-1}$ in buffer 3837 ± 37 M$^{-1}$ cm$^{-1}$ in dioxane.

7,8-dinitro-1,3-diaza-2-oxophenoxazine-2'-deoxy-5'triphosphate-β-D-ribofuranoside.

Absorbance spectra were obtained with the source changing over set to occur at 350 nm which is the visible jump in the spectra. Anthracene diluted to concentrations of 15, 10, 6, 5, 4, 3, 2, 1 and 0.5 μM in ethanol were used in comparison with 7,8-NO2-tCo in 1X PBS.
buffer at concentrations of 10, 6, 5, 4, 3, 2, 1 and 0.5 μM. Absorbance maxima of 280 ± 1 nm in buffer. Absorptivity of 18600 ± 12210 M⁻¹ cm⁻¹.
Fluorescence of Oxygen tricyclic cytosine analogs

A large Stokes shift is desirable because the further red shifted the emission the more useful a probe will be in biological applications. In these experiments tC° was observed to have a fair Stokes shift of 105 nm in buffer landing the emission wavelength at 460 nm which is slightly blue shifted compared to tC and compared to the value reported in the literature.37 The sulfur version of this molecule has a preferable Stokes shift of 126 nm landing emission at 503 nm. Both of these parent compounds were observed to have a blue shift when placed in a less polar environment. Sulfur skeleton tC emission maxima falls to 472 nm in dioxane and tC° slides a lesser 18 nm in this solvent. The quantum yield is more favorable in the opposite solvent to that which has the larger Stokes shift. 0.217 higher quantum yield is seen in dioxane for tC° and 0.251 higher is the quantum yield for tC in this environment. Upon incorporation there is a slowing of the nonradiative decay processes, which allow for an increase in QY of this FBA when away from the aqueous solvents. The nonradiative decay of these parent compounds being equal, it is assumed that the increase in the fluorescence comes from an increase in the fluorescence rate constant which comes from increased oscillator strength.61 Below is a table of the fluorescence properties of the analogs tested.101

Table 6: Fluorescence of Analogs are presented with wavelengths of emission and Stokes shift in nm, and brightness in M⁻¹ cm¹.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>λ Em</th>
<th>Φ</th>
<th>Stokes</th>
<th>ϕE</th>
</tr>
</thead>
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<td>74</td>
<td>1400</td>
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<tr>
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<td>108</td>
<td>570</td>
</tr>
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</table>
The presence of the fluorine did not have a great effect in either of the environments on shifting the emission maxima. A slight blue shift of 10 nm in emission wavelength was seen for 8-F-tC from buffer at 462 nm to 452 nm in dioxane. The quantum yield was little changed (12-25 percent) with this substitution as well, differing from the parent compound by only 0.053 and 0.069 with the greatest difference coming in an aqueous environment. The presence of fluorine in the major groove has been shown to not have rotation restricted. Rotation effects on quenching will be mentioned later in discussion of the methoxy analog, but a lack of quantum yield difference is interesting.

This lack of influence observed for the fluorine substitution is in comparison to the tetracyclic analog which shifts the emission wavelength in buffer 26 nm to 434nm but only 4 nm in dioxane. Due to the previously discussed decrease in the HOMO-LUMO gap from extended aromaticity the decrease in Stokes shift is not surprising for tetC. The solvent dependence of this decrease is perhaps explained better by examination of the parent compound because the Stokes shift difference between the solvents for tetC is only 2nm. Vibrational modes are used to explain the photophysical behavior of tC. Visibility of the distinct transitions shows different energy levels are likely being
populated. Quantum yield for this analog was more environmentally sensitive, and was higher than that for the parent compound in buffer but lower in dioxane by almost the same amount.

**Figure 4-0-9:** Emission spectra of tC\textsuperscript{o} analogs. Spectra obtained in dioxane and in buffer have been normalized to emphasize spectral shape and maximum wavelength.

TetC\textsuperscript{o} had the greatest observed decrease in Stokes shift in an aqueous environment, and the greatest increase was seen with the methoxy substituents. An explanation for this phenomenon could be greater stability of the excited state provided by this electron donating group. This pattern holds true for both the sulfur and the oxygen containing analogs. 7-OMe-tC\textsuperscript{o}, 8-OMe-tC\textsuperscript{o}, 8-OMe-tC have emission wavelength differences of 27, 26, 35 nm from the parent compounds respectively. The shifts are in the same direction but not to as large an extent when the compounds are compared in dioxane. 7-OMe-tC has a slight shift from the parent compound in either solvent which is surprising given that the 8-OMe-tC had the most significant influence on emission wavelength. If the substitution causes the excited and ground state geometries to be more similar, this would have a greater effect on tC versus tC\textsuperscript{o} because it has a greater difference to overcome.\textsuperscript{61} Stokes shifts are 2-15 nm larger for these analogs in this non-hydrogen bonding solvent. Methoxy substituents caused twice the decrease in quantum
yield for analogs in buffer compared to experiments in dioxane. but they consistently decrease the emission of the compounds. Opposite to the influence on wavelength, the decrease in quantum yield from these ether groups was strongest on the oxygen framework. Fair (45-90%) 0.041 and 0.081 decreases were observed for 7 and 8-OMe-tC respectively in phosphate buffer experiments. Modifications that allow for rotation create competition for fluorescence in the form of torsional relaxation, and this is a potential explanation of the quenching observed by the presence of the ether substitution. With tC\textsuperscript{o} having the greater fluorescence rate constant, the substitution influence on decay processes may have a more significant impact on the oxygen framework. Although 8-Cl-tC\textsuperscript{o} shifts the emission 25 nm to 417 nm in dioxane it only has a Stokes shift difference of 11 nm compared to the parent compound in buffer. When this substituent is in the para position (7-Cl-tC\textsuperscript{o}) there is almost no difference in emission wavelength in buffer and a slight 6 nm change from tC\textsuperscript{o} in dioxane. Both analogs which contain this heavy atom show a change in quantum yield of greater than 0.1 in phosphate buffer. These quantum yield enhancements from the chlorine do not translate to nonpolar environments with 0.031 and 0.001 change in comparison to the parent in dioxane. This is interesting when compared to the literature which suggests that heavy atoms promote intersystem crossing. Other explanations have been offered to explain halogen’s enhancement of fluorescence, such as an increase in fluorescence lifetime which increases the quantum yield. Below are the graphs for Hammett constant correlation for fluorescence characteristics.
Figure 4-0-10: Emission correlation between substituent Hammett constant and analog emission wavelength. Change refers to the difference in the photophysical property as compared to the unsubstituted parent compound. No linear relationship is seen with substitution at position 7 of the tricyclic framework but linear relationship seen between substitution at position 8 in either dioxane or buffer.
Figure 4-0-11: Emission correlation between substituent Hammett constant and analog quantum yield. Linear relationships are seen with substitution at positions 7 and 8 with stronger correlation at the 7 position for analogs in dioxane and at the 8 position for analogs in buffer. Change refers to the difference in the photophysical property as compared to the unsubstituted parent compound.
The emission wavelength correlates with substitution at the 8 position with a more linear relationship for measurements made in buffer versus those in dioxane. In buffer the + Hammett constant is closest match to the substituent effect on emission wavelength. Contrarily, in dioxane the – Hammett constant relates strongest to the effects that the substitutions had. Differences in Stokes shift in buffer are clearly more correlated with this same position on the chromophore which places the substituent para to the second ring nitrogen. In dioxane there is a fair correlation to either position. Quantum yield has a similar closeness in correlation with both positions in the different environments. The 8 position relate slightly better in buffer and the 7 position is closer in dioxane. Opposite to the emission wavelength Hammett constants, the + Hammett value is closest in dioxane and the – in buffer.

The product of emission quantum yield and absorptivity is a quality termed brightness. A 768 M⁻¹cm⁻¹ increase in brightness was observed for 8-Cl-tC⁰ in dioxane followed closely by 7-Cl-tC⁰ in buffer. Buffer measurements are useful because even though this is the free nucleoside, and the environment within nucleic acids is sheltered from the solvent, this is the media in which double and single stranded DNA are likely to be studied. The greatest decrease 2399 M⁻¹cm⁻¹ is seen by 7-OMe-tC in dioxane. Solvent susceptibility as will be discussed later can explain some of what is seen, the overall trend in brightness with compounds in dioxane is 8-Cl-tC⁰>7-Cl-tC⁰>tC⁰>tetC⁰>8-OMe-tC⁰>tC>8-Fl-tC⁰>8-OMe-tC>7-OMe-tC. In buffer the overall trend is 7-Cl-tC⁰>8-Cl-tC⁰>tC⁰>tetC⁰>tC>8-Fl-tC⁰>8-OMe-tC⁰>7-OMe-tC>7-OMe-tC>8-OMe-tC. Brightness correlation with Hammett values is presented below.
Figure 4-0-12: Brightness correlation between substituent Hammett constant and analog brightness (product of quantum yield and absorptivity). No linear relationship is seen with substitution at position 8 of the tricyclic framework and slight linear relationship is seen between substitution at position 7 in either dioxane or buffer. Change refers to the difference in the photophysical property as compared to the unsubstituted parent compound.
Brightness correlates best with substitution at the 7 position on the nucleoside, in either solvent, but this deviates somewhat from linearity for electron donating substituents. With the many factors affecting each property, it makes sense that a strong relationship between Hammett values and this photophysical property is not evident. In dioxane, the + and – constant values relate almost equally to the data for changes in brightness.

Although there is no fine structure apparent in the absorbance spectra of the tC analogs following smoothing, there does appear to be some shouldering on either side of the emission maximum when looking at the emission of these analogs in buffer. This shouldering is indicative of vibrational levels of the excitation. Shouldering is less apparent when the tC compounds are in dioxane, and this is the opposite of the trend that is seen with the oxygen analogs. 8-F-tC® and the other meta position substituents show some fine structure like the unsubstituted tC® analog in buffer but this is much more intense in dioxane. Other FBAs have been shown to have single fluorescence decays as free molecules in solution that become an ensemble in higher order structures due to the different environments that are encountered.

The dinitro analog shows significant loss of fluorescence, which was also observed with the addition of a single nitro substituent onto the sulfur skeleton. This decrease has been attributed to vibrational deactivation, because it is temperature dependent and at a frequency which indicates an internal conversion process. There have been two calculated low energy coordinates for the tC nitro compound on the potential energy surface of this compound. It has also been hypothesized that quenching from
phosphate can occur as a kinetic process. The presence of the phosphate groups alters the $pK_a$ of the nucleobase, and this may have some influence on the photophysics that were observed as well.

7-OMe-tC and tetC$^0$ were shown to be the most environmentally sensitive in the Reichardts dye (RD) study discussed in the introduction to this chapter. However these both have differences in brightness between buffer and dioxane that are less than that of the parent compound and many of the other members of the family. 7-OMe-tC has the smallest decrease in brightness when going from dioxane to buffer out of all the analogs tested 474 M$^{-1}$cm$^{-1}$. The analog that was seen to have a large change in brightness between the environments of 2204 M$^{-1}$cm$^{-1}$ was the 8-Cl-tC$^0$ analog. All other analogs show a brightness sensitivity that is less than that of the parent compounds. However because of the limited change in Stokes shift, this analog tested very insensitive in the RD experiment measuring polarity sensitivity. Viscosity differences in the solvent mixtures have been used to explain the behavior of analogs which have rotor like appendages, and therefore are subject to the effects of the viscosity of the solvent as well. In the Reichardts dye experiment measuring solvent sensitivity it was seen that 8-Cl-tC$^0$ was the least sensitivity to polarity, followed by 7-OMe-tC$^0$. Although not the most different in brightness between the two solvents, this para methoxy analog has one of the most significantly different quantum yields between dioxane and buffer. The smallest change in Stokes shift between the two solvents was seen with the tetC$^0$, but it has a change in brightness on the larger side of the spectrum. In previous work it has been shown that combinations of twisted intramolecular charge transfer and conformational effects have
been used to explain these strange observations. Hydrogen bonding may stabilize a certain conformation over another which results in the observed changes in the photophysics. Environmental sensitivity needs to be further studied for the analogs but sensitivity or lack thereof can be useful. Those analogs that do show some sensitivity to their environment can report dynamics information while those that are not can maintain fluorescence through environment changes.
Fluorescence Experimental:

Fluorescence spectroscopy was performed using a Varian Cary Eclipse Fluorescence Spectrophotometer. Emission spectra were obtained using a wavelength range of 15nm past the excitation wavelength to 600 or 700nm, an averaging time of 0.100s with a data interval of 1 nm. The scan rate was 600 nm per minute with excitation and emission slit widths of 5nm and a medium PMT voltage with no applied correction. A baseline signal of the solvent being used was taken prior to sample collection and subtracted from the data acquired. Correction data were not available for the entire wavelength range of interest, so they were developed by the comparison of the emission intensity of 1,1,4,4-tetraphenyl-1,3-butadiene, Coumarin 153, and 4-(dicyanomethylene)-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran to standard literature values. The emission spectra were then integrated and the integrations used in comparison with quinine sulfate standard and absorption data above to give quantum yield. Samples were taken from a stock 6mM solution of each of these analogs and diluted to desired concentrations with the solvent for investigation. A sample would be measured for absorbance and emission in the same cuvette (which was marked for repetitive orientation) at the same time. 1X PBS buffer was made from dissolution of phosphate buffered saline tablet in millipure water. One tablet in 200 mL of water gives 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. 0.1 M H₂SO₄ was made from dilution of concentrated sulfuric acid with water from a Millipore filtration system. Spectroscopic grade dioxane was used. Quinine sulfate standards were made from dissolution of a measure of quinine hemi-sulfate in DMSO to
create a stock 6mM solution. Average emission wavelengths from 6-13 measurements are reported with standard deviations. Average quantum yields from 2 measurements are reported with standard deviations to represent half of the difference between the measurements.

**tC** 2'-deoxy-β-D-ribonucleoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tC in 1X PBS buffer at concentrations of 22, 20, 18, 16, 14, and 12 μM or 22, 21, 20, 18, 16, and 12 μM or 24, 20, 12, 8, and 4 μM and in dioxane at concentrations of 22, 20, 18, 16, 14, and 12 μM or 22, 20, 16, 12, 8 and 4 μM. Emission maxima of 503 ± 4 nm in buffer 472 ± 2 nm in dioxane. Quantum Yields of 0.093 ± 0.002 in buffer 0.344 ± 0.016 in dioxane.
1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tCo in 1X PBS buffer at concentrations of 14, 12, 10, 8, 6, and 4 μM and in dioxane at concentrations of 12, 10, 8, 6, 4, and 2 μM in duplicate. Emission maxima of 460 ± 4 nm in buffer 442 ± 2 nm in dioxane. Quantum Yield of 0.244 ± 0.016 in buffer 0.461 ± 0.030 in dioxane.

1,3-diaza-2-oxophenoxyzine-2'-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with tetC⁰ in 1X PBS buffer at concentrations of 20, 18, 16, 14, 12, and 10 μM or 20, 16, 12, 8, 4, and 2 μM and in dioxane at concentrations of 12, 10, 8, 6, 4, and 2 μM in duplicate. Emission maxima of 434 ± 2 nm in buffer 438 ± 1 nm in dioxane. Quantum Yield of 0.270 ± 0.016 in buffer 0.433 ± 0.002 in dioxane.
7-chloro-1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-Cl-tC₀ in 1X PBS buffer at concentrations of 12, 10, 8, 6, 4, and 2 μM and in dioxane at concentrations of 10, 8, 6, 4, 2, and 1 μM in duplicate. Emission maxima of 456 ± 4 nm in buffer 446 ± 1 nm in dioxane. Quantum Yield of 0.355 ± 0.032 in buffer 0.460 ± 0.022 in dioxane.
*8-chloro-1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside.* Quinine Sulfate diluted to concentrations of 18, 16, 14, 12, 10, 8, 6, and 4 μM or 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-Cl-tC⁰ in 1X PBS buffer at concentrations of 14, 12, 10, 8, 6, and 4 μM and in dioxane at concentrations of 12, 10, 8, 6, 4 and 2 μM. Emission maxima of 455 ± 3 nm in buffer 417 ± 40 nm in dioxane. Quantum yields of 0.0349 ± 0.041 in buffer 0.492 ± 0.034 in dioxane.
**7-methoxy-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-OMe-tC⁹ in 1X PBS buffer at concentrations of 42, 34, 26, 18, 10, and 2 μM or 36, 30, 24, 18, 12, and 6 μM or 25, 20, 15, 10, 5, and 2.5 μM and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 34, 26, 18, 10, and 2 μM. Emission maxima of 487 ± 6 nm in buffer 449 ± 4 nm in dioxane. Quantum Yield of 0.053±0.001 in buffer 0.369 ± 0.006 in dioxane.
**8-methoxy-1,3-diaza-2-oxophenoxazine-2'-deoxy-β-D-ribofuranoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H$_2$SO$_4$ were used in comparison with 8-OMe-tC$_0$ in 1X PBS buffer at concentrations of 24, 20, 16, 12, 8, and 4 μM and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 18, 15, 12, 9, 6, 3 μM or 18, 14, 10, 8, 6, and 3 μM. Emission maxima of 486 ± 6 nm in buffer 449 ± 2 nm in dioxane. Quantum Yield of 0.050 ± 0.000 in buffer 0.380 ± 0.012 in dioxane.
8-fluoro-1,3-diaza-2-oxophenoxazine-2’-deoxy-β-D-ribofuranoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-F-tC° in 1X PBS buffer at concentrations of 80, 70, 60, 50, 40, and 30 μM or 50, 40, 30, 20, 10, and 5 μM and in dioxane at concentrations of 42, 34, 26, 18, 10, and 4 μM or 50, 40, 30, 20, 10, and 5 μM. Emission maxima of 462± 2 nm in buffer 452 ± 2 nm in dioxane. Quantum Yield of 0.175 ± 0.006 in buffer 0.408 ± 0.000 in dioxane.
**7-methoxy-tC 2’-deoxy-β-D-ribonucleoside.** Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 7-OMe-tC in 1X PBS buffer and in dioxane at concentrations of 25, 20, 15, 10, 5, or 2.5 μM in duplicate. Emission maxima of 508±14 nm in buffer 477±2 nm in dioxane. Quantum Yield of 0.052 ± 0.001 in buffer 0.266 ± 0.004 in dioxane.
8-methoxy-tC 2’-deoxy-β-D-ribonucleoside. Quinine Sulfate diluted to concentrations of 14, 12, 10, 8, 6, and 4 μM in 0.1 M H₂SO₄ were used in comparison with 8-OMe-tC in 1X PBS buffer at concentrations of 24, 20, 16, 12, 8, and 4 μM or 30, 23, 15, 11, 7, and 3 μM in duplicate and in dioxane at concentrations of 36, 34, 32, 30, 28, and 26 μM or 24, 20, 16, 12, 8, and 4 μM. Emission maxima of 538 ± 3 nm in buffer 490 ± 2 nm in dioxane. Quantum Yield of 0.011 ± 0.000 in buffer 0.277 ± 0.014 in dioxane.
7,8,-dinitro-1,3-diaza-2-oxophenoxazine-2'-deoxy-5' triphosphate-β-D-ribofuranoside. Emission spectra were obtained with the source changing over set to occur at 350 nm which is the visible jump in the spectra. Anthracene diluted to concentrations of 15, 10, 6, 5, 4, 3, 2, 1 and 0.5 μM in ethanol were used in comparison with 7,8-NO2-tCo in 1X PBS buffer at concentrations of 10, 6, 5, 4, 3, 2, 1 and 0.5 μM. Emission maxima of 389 nm in buffer. Quantum Yield of 0.03.
Solvent Sensitivity Experimental:

Mixtures of 10, 30, 50, 70, and 90 dioxane in 1X phosphate buffered saline were prepared. The analogs were made into samples on 30μM that have less than 0.4 percent by volume of DMSO from the stock solution. Absorbance and emission spectra were taken of the nucleosides and Reichardts dye in the above mixtures. Emission spectra were corrected and the maximum wavelength in combination with data from the quantum yields studies for the pure solvents and the maximum absorbance wavelength used to determine a Stokes shift in wavenumbers. These values were the y axis values and the Et(30) values for Reichardts dye in the corresponding solvent mixtures were the x axis values for the plots presented below. The slopes of the lines of these graphs were used as a measure of environmental sensitivity for the compound under study.

8-chloro-1,3-diaza-2-oxophenoxazine 2’-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30)value (the shift in absorbance value for Reichardts dye).
**tC 2′-deoxy-β-D-ribonucleoside.** On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

**1,3-diaza-2-oxophenoxazine 2′-deoxy-β-D-ribofuranoside.** On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).
1,3-diaza-benzo(h)2-oxophenoxazine 2’-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

7-chloro-1,3-diaza-2-oxophenoxazine 2’-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

7-methoxy-1,3-diaza-2-oxophenoxazine 2’-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).
8-methoxy-1,3-diaza-2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

8-fluoro-1,3-diaza-2-oxophenoxazine 2'-deoxy-β-D-ribofuranoside. On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).
**7-methoxy-tC 2’-deoxy-β-D-ribonucleoside.** On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

**8-methoxy-tC 2’-deoxy-β-D-ribonucleoside.** On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Stokes shifts in wavenumbers as a function of the polarity of the solvent. Polarity was defined as the Et(30) value (the shift in absorbance value for Reichardts dye).

**Reichardts dye** On the left are the spectra for the compound obtained in dioxane and water and mixtures thereof. On the right are the plots of the Et(30) value (the shift in absorbance value for Reichardts dye) and percent of buffer.
Stokes shift correlation between substituent Hammett constant and analog absorbance and emission wavelength separation. No linear relationship is seen with substitution at position 7 of the tricyclic framework and linear relationship is seen between substitution at position 8 in buffer only. Change refers to the difference in the photophysical property as compared to the unsubstituted parent compound.
Chapter 5 Biological Activity of Analogs

Introduction

Fidelity of nucleic acid replication depends on the ability of the enzyme to select and incorporate the correct nucleotide. Error rates vary between $10^{-3}$ and $10^{-6}$ errors per nucleotide incorporated when the enzyme cannot proofread and $10^{-8}$ and $10^{-11}$ when it can. There is an intrinsic yet minor natural mechanism to permit purposeful mutations, through errors in replication, for evolution. Watson and Crick were the first to propose that this mechanism may rely on the tautomerism of the nucleotides. Uridine and thymidine are likely to be found in the diketo form, while the keto-amino form is predominant in cytosine and guanine, with adenine alone adopting the amino form as the major tautomeric configuration. The presence of the sugar has been shown to prevent some tautomerism in nucleobases. Placement of the nucleobase nitrogen in the glycosidic bond limits its participation in the tautomers that would be accessible by the free base. Because the tautomeric form of the base can influence photophysics, understanding the tautomeric equilibrium of a nucleotide can help explain its chromophore capabilities. The fluorescence decay has been shown to be different between the nucleobase and the nucleoside which is not entirely unexpected. Even the nucleoside and the nucleotide have differing decays which is surprising given they differ only by presence of phosphate. Tautomerism affects more than just the photophysics that were presented in
Chapter 4, so the positions of nucleobase protons, dictated by tautomeric equilibria of the analogs, is under investigation.

![Diagram of tautomeric forms](image)

Figure 5-0-1: Tautomerization of analogs. These are two of the tautomeric forms for the substituted tricyclic cytosine analogs investigated using incorporation and low temperature NMR.

Experiments by collaborators at the University of Colorado at Boulder have been published characterizing incorporation of the parent compounds tC and tC°. This chapter presents the characterization of the substituted analogs. These early experiments reveal that the dinitro substituted tC° analog, the synthesis of which was presented in Chapter 3, can incorporate more efficiently than the natural nucleotides opposite either G or A. Other analogs are awaiting analysis for incorporation fidelity by polymerases. The substitutions will likely have an influence on the tautomeric equilibrium between the amino and the imino forms of cytosine because of their varying electronic and resonance contributions. Substituting bromine onto a uracil changes its tautomeric equilibria and its pairing abilities. It is proposed that this substitution increases the occurrence of the enol form of the compound by an order of magnitude.

Another synthetic nucleotide 2AP has been shown to pair with C or U in the wobble configuration while finding a partner in T with natural hydrogen bonding. X-ray structures have shown the presence of wobble base pairs in short duplexes. Tautomeric mismatches can resemble the size and the shape of the natural base pairs. Wobble pairs which result from ionization of a base do not resemble the natural system.
as closely. This decreases the likelihood that wobble pairs are the mechanism for evolutionary mutations. Tautomer pairs are more likely to make it through the five polymerase check points than the wobble pairs due to a closer resemblance to the Watson-Crick pair. These check points are the pre-insertion site, the insertion site, the catalytic site, the post-insertion site, and the duplex binding region, which all verify that the correct base has been inserted. Stabilization of these higher energy tautomeric pairs is achieved by hydrogen bonding with a water molecule that is found in the polymerase active site. Additional stabilization comes from the electrostatic field. Mismatch as a result of a tautomerism can also be stabilized by a cofactor that binds to the enzyme and encourages maintenance of the closed state of the polymerase enzyme. \(^{146}\) NMR has been used to study the tautomeric CA pair. \(^{64}\)

A change in the metal cofactor from Mg\(^{2+}\) to different metals was shown to be helpful to the tautomeric base pairs in the polymerase active site. Nickel was shown in a study of guanine to inhibit tautomerism. Association with nickel prevents formation of the lowest energy G7 tautomer. Coordination between the metal and the analog occurs at the 7 position. In the absence of this metal, the authors had been able to use heat to induce guanine tautomerization from the normal G9 tautomer to the G7 (figure 5-0-2). \(^{147}\) Guanine is not only the easiest to oxidize, but it has been suggested to be the nucleobase that is easiest to tautomerize. \(^{148}\) Computational studies into the C-G tautomeric base pair revealed that tautomerism, cis-trans isomerism, and steric all play a role in the stability of the tautomeric pair. Tautomerism plays interesting roles in the behavior of nucleic acids in the presence of metals as well as in the formation of triplex structures. \(^{149}\)
Mutagenesis and carcinogenesis have been observed as a result of O6 alkylation of G. The G to A mutation may come from the preferred pairing of this alkylated guanine with T instead of C. There is no difference in the stability of this duo, but it has an effect on the overall stability of a duplex containing this pair. The decrease in duplex stability may be a steric problem, because sequence dependent melting temperatures were not observed. The same steric issue could play a role in the polymerase active site explaining the observed G to A mutation but it also could be a tautomeric effect.

Tautomeric base pair mismatches have been used as a means to control the cellular functions of some synthetic nucleotides. A photoinduced change in the hydrogen bonding face was shown to be a means of controlling recognition and complementarity of an analog by cellular elements. Site specific irradiation could be used to change the partner of an analog from guanine to adenine. Some applications make the lack of pairing specificity desirable. An analog that is useful for this purpose is N-alkoxy cytosine which can tautomerize to pair as either T or C. These are preferable to analogs that stack but don’t hydrogen bond because of the increased stability.

Tautomerism remains an understudied phenomenon of nucleosides, because the availability of sensitive techniques to study the tautomeric forms of nucleotides are limited. Interconversion of tautomers can occur in less than a millisecond. Through the use of a combination of techniques, it was recently possible to study tautomeric...
recognition by a riboswitch. Tautomerism is of interest in terms of antivirals because nucleotides with tautomeric forms can accelerate mutation rates of RNA viruses to extinction.\textsuperscript{152}

Due to the tunneling effect, quantum jumps of protons can occur, which is a proposed method for formation of a different tautomeron of a nucleobase. If this happens in one strand it can encourage the nucleobase in the complementary strand to tautomerize to balance the hydrogen bonding pattern. If ionization due to proton jumping without tautomerization occurs this would lead to deletions because there is no partner to be found for replication.\textsuperscript{153}

Compartmentalized self-replication is a polymerase evolution strategy that results in enzymes with a more tolerable active site. Short patch versions of this replication have only a small defined sequence of the gene replicated resulting in enzymes with the active site but not other active domains, i.e. those for proofreading. This has been utilized with synthetic nucleotides, because it makes possible the enzymatic incorporation of normally intolerable nucleotides. B family polymerases, evolved with a diversification in the A mobile and the C mobile regions of the active site, have been used for incorporation of synthetic nucleotides. “Specialist” polymerases were also found which would select for dye labeled nucleotides and incorporate them more efficiently than the natural nucleotides. Polymerases have an error threshold, and those that are too error prone will not be functional at all, so there are limits to this technique. The minor groove is where the hydrogen bonding between the nucleic acids and the polymerase enzyme takes place.\textsuperscript{9}

By starving the polymerase enzyme of specific triphosphates, replication can be stalled at
specific sites. This has been used to produce fluorescent snapshots with fluorescent nucleotides of the enzyme DNA complex. ⁴¹
Dinitro Incorporation

Using steady state conditions, it is possible to use polymerization of the analogs synthesized in Chapter 3 to determine the kinetics and fidelity of the analogs with biological machinery. Phosphorous labeled template strands make visualization of the incorporation products possible following denaturing electrophoresis. Tolerance of the substituted analogs is important in their utility as biophysical probes. Additionally the ambiguous characterization of the presence of phosphate functional groups on the deoxyribose of these compounds is verified by the ability to be an enzyme substrate for synthesis.

Table 7: Kinetic incorporation comparison between 7,8-NO₂-tC°-dTP and natural nucleotide partners for polymerization opposite A and G in the 13th position of a 25 base strand sequence.

<table>
<thead>
<tr>
<th>dNTP</th>
<th>Template</th>
<th>Vₘₐₓ</th>
<th>Kₘ</th>
<th>Vₘₐₓ/Kₘ</th>
<th>Discrimination</th>
</tr>
</thead>
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<td>2.47</td>
<td>0.32</td>
<td>7.70</td>
<td>1</td>
</tr>
<tr>
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<td>2.38</td>
<td>0.25</td>
<td>9.48</td>
<td>0.5</td>
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<td>0.19</td>
<td>3.59</td>
<td>1</td>
</tr>
<tr>
<td>7,8-NO₂-tC°-dTP</td>
<td>DNA₉</td>
<td>2.93</td>
<td>0.38</td>
<td>7.70</td>
<td>0.8</td>
</tr>
</tbody>
</table>

7,8-NO₂-tC°-dTP was tested for its incorporation kinetics. Tricyclic cytosine analogs incorporate across from A and G as was previously established. Therefore, this substituted analog was tested for incorporation against both of these templating bases. As discussed in the introduction, it is hypothesized that the ability to pair with both of these natural bases comes from a tautomeric ratio between the amino and imino forms that is not as one sided as that for cytidine.
Incorporation by Klenow fragment polymerase onto the primer opposite adenine in the template sequence resulted in an observed V_max of 2.38 (percent/min) for 7,8-NO_2-tC^o and 2.47 (percent/min) for thymidine (figure 5-0-3, table 7). These faster kinetics for the unnatural base analog were not seen in the experiments with the parent compounds tC and tC^o. Both of these unsubstituted skeletons had V_max for incorporation opposite adenine that were higher than that for natural cytosine, but lower than thymidine. This means that the maximum rate of the incorporation reaction is greater for this new analog than for the natural nucleotide pair. This measure of the differences in the catalytic rate constants is complicated because of the multiple steps involved in polymerase activity.\textsuperscript{154}

Faster kinetics were observed not only for the dinitro compound as a substitute for T in pairing with A, but also in the place of C pairing with G. Using the same primer sequence and a template with only a single nucleotide difference, the new analog was tested for polymerization. Incorporation opposite guanosine was seen to have the greatest V_max of 2.93 (percent/min) while the natural C was shown to have a 0.69 V_max under the conditions tested (figure 5-0-4, table 7). Tricyclic cytosine and tC^o have shown to have faster kinetics than the natural cytosine but not nearly to the extent observed for 7,8-NO_2-tC^o.\textsuperscript{64}
As a measure of the dissociation of the polymerase-DNA-nucleotide complex, the 
$K_M$ is a representation of the affinity in the enzyme substrate complex for the incoming 
nucleotide.\textsuperscript{7,154} The $K_M$ for the enzyme substrate complex where adenine is templating is 
0.25 μM with 7,8-NO$_2$-tC$^0$-dTP which is lower than the 0.32 μM seen for the natural 
binding partner. A lower concentration of the nucleotide is needed to reach half saturation 
of the enzyme that is present which could be evidence of a greater affinity of the enzyme 
for the modified base.

The reverse is seen when incorporating opposite guanosine. Cytidine has a $K_M$ in 
the assay of 0.19 μM and 7,8-NO$_2$-tC$^0$ was 0.38 μM. This decrease in enzyme affinity as 
compared to the natural nucleotide was seen for the parent compounds in incorporation 
opposite both A and G in previous work.\textsuperscript{64}

Combining the two kinetic parameters gives a measure of overall incorporation 
efficiency through examining how the polymerase functions at low nucleotide 
concentrations for the natural base versus the synthetic base. $V_{\text{max}}/K_M$ of 7,8-NO$_2$-tC$^0$ is 
9.48 (percent min$^{-1}$ μM$^{-1}$) versus 7.70 (percent min$^{-1}$ μM$^{-1}$) for thymidine with regards to 
incorporation with the templating adenine. Greater efficiency of 7,8-NO$_2$-tC$^0$ is observed 
across both templates. With guanosine as a partner, $V_{\text{max}}/K_M$ is 7.70 (percent min$^{-1}$ μM$^{-1}$) 
for the substituted tricycle and 3.59 (percent min$^{-1}$ μM$^{-1}$) for the natural version of 
cytosine. The fidelity of the tC and tC$^0$ is reported to be equal to that of the cytosine and 
less than that for thymidine with their respective natural partners.

Enzyme discrimination is the ratio of $V_{\text{max}}/K_M$ for the natural nucleotide versus 
the analog. Less discrimination (0.5) than that for the natural analog (1) was seen for 7,8-
NO\textsubscript{2}-tC\textsuperscript{o}, as well as tC (0.6) and tC\textsuperscript{o} (0.6) when incorporating opposite guanosine. However, the discrimination of (0.8) for the dinitro compound with regards to incorporation opposite adenosine is thus far unique for the tricyclic cytosine family, the parent compounds and cytosine have values greater than 1.\textsuperscript{64}

**Dinitro Tautomerism**

In the NMR spectrum of the substituted tricyclic cytosine analogs there is an interesting broadness at around 7.7 ppm in the peaks of the nucleobase. An investigation was made into whether this broadness would resolve into two peaks at low temperature, perhaps representing the two tautomeric forms of the nucleobase that would explain the incorporation behavior seen above.

![NMR Spectra](image)

**Figure 5-0-3: 7,8-NO\textsubscript{2}-tC\textsuperscript{o} in DMSO. NMR spectra presented for the proton signals of the nucleoside.**

Low temperature NMR studies were not possible in DMSO (which is the best solvent for obtaining spectra of the nucleosides). To investigate the possible solvents to use for this study, deuterated acetone and dichloromethane were tried. Limited solubility in dichloromethane eliminated that solvent immediately. In acetone the solubility was less than in DMSO, but it was soluble enough for measurements to be attempted. Spectra were obtained from 30 °C down to -80 °C. Unfortunately, as the temperature decreased the low solubility became an issue, and the peaks for the nucleoside were difficult to follow. There was some apparent movement of the aromatic proton signals, but no discernible resolution of one peak into two.
To improve the solubility, spectra were taken in a mixture of 40 percent deuterated DMSO with 60 percent deuterated chloroform. Spectra were obtained from 30 °C down to -30 °C. Broadening not seen in the mixture of solvents at room temperature appears in the aromatic region at lower temperature, but NMR was not able to be measured at temperatures low enough to resolve the tautomeric forms.

To confirm the assignment of the exchangeable protons, a small amount of deuterium oxide was added to a sample in the above mixture at 30 °C. This resulted in a change in two peaks which were suspected to be NH protons (see experimental). Finally, to ensure the peaks that were assigned in DMSO were the same as those in this mixture a titration of deuterated chloroform into a sample of the 7,8-NO2-tC° in deuterated DMSO was performed. Upon addition of the first hundred microliters of chloroform, the coupling is disturbed and broadening of peaks was observed. After four hundred microliters the peaks sharpen to those seen in the sample used for the low temperature measurements (figure 5-0-6).
Figure 5-0-4: Titration of chloroform into DMSO. 100μL portions of CDCl₃ (2-6) were added to a sample of the 7,8-NO₂-tC₈ in DMSO (1) to establish which signals in a mixture of solvents correspond to the proton signals in DMSO.
**Dinitro Experimental:**

Single nucleotide insertion assays were used to determine $K_M$ and $V_{\text{max}}$. Experiments were performed with 0.5 nM Klenow fragment polymerase enzyme, 2 μM primer-template, 2, 5, 10, 20, 40 and 500 μM 7,8-NO$_2$-tC$^o$-dTP, 0.5, 0.75, 1, 2, 4, and 6, μM dTTP, or 2, 5, 10, 20, 40 and 500 μM dCTP with 5 mM MgCl$_2$, 50 mM Tris-HCl pH 7.5 in a total volume of 10 μL. The DNA primers were $^{32}$P labeled on the 5’ terminus using T4 polynucleotide kinase prior to being annealed to the template strand. Assays were stopped at 2.5 and 10 minutes by addition of two volumes gel loading buffer (90% formamide with 50 mM EDTA. Incorporation products were separated by denaturing gel electrophoresis (20% polyacrylamide, 8 M urea) and analyzed by phosphorous imaging. Nonlinear curve fitting was used to derive the kinetic parameters presented.

Saturated Samples of 7,8-NO$_2$-tCo were prepared in corresponding deuterated solvents for variable temperature NMR. Spectra were collected on an INOVA 500 MHz spectrometer. Titration and proton exchange experiments were collected on a VNMRS 400 MHz spectrometer.
Variable temperature of 7,8-NO₂-tC° in acetone. Measurements were made from 30 degrees (1) down to -80 degrees (-10) but the limited solubility of the nucleoside made determination of the proton signals at lower temperatures difficult.
Variable temperature of 7,8-NO2-tCo in 40% DMSO, 60% CDCl3. Measurements were made from 30 degrees (1) down to -30 degrees (7). Experiments were looking for a resolution of the two amino proton signals for the tautomeric forms of the nucleoside which were not seen.

Proton exchange of 7,8-NO2-tCo. Deuterium oxide was added to confirm the proton signals for exchangeable hydrogens of the nucleoside. The 5' and 3' hydroxyl signals at around 5 ppm decreased as well as the potential amino proton signals at around 11.5 ppm and 7.7 ppm.
Chapter Six: Conclusions and Future Work

The overarching correlation between all experiments in this work is the effect of substituents; they affect photophysics, synthesis, and incorporation. As discussed in the fourth chapter of this work, there are some correlations between the photophysical properties and the substituents that were synthesized into the chromophore framework. Correlation with quantum yield is not position dependent whereas influence on absorptivity by substitution is affected by location. Absorbance wavelength changes are not linearly related to Hammett constant but emission wavelength differences are. Observed reactivity of compounds in the synthesis presented in chapters two and three correlates with the Hammett constants of the substituents as well. The most unreactive compound towards secondary amine formation was the 4-chloro-2-aminophenol, and this has the greatest Hammett constant (meta to amine formation). Ring closure was difficult with the secondary amine formed from 5-chloro-2-aminophenol which has the same Hammett constant (meta to ether formation). Character of the substituent explains the enzymatic fidelity presented in chapter five as well. The effect of substitution was effectively examined through a series of closely related compounds, but more remains to be gleaned from this small library because in general, the trends do not fully explain all of the results. Sometimes electronic affects are more important and in other cases resonance substituent character has a greater influence.
Nucleic acid studies of the compounds may shed some light on the questions of which analogs are the most and least sensitive to their environment. Conflicting results obtained from the nucleosides may be resolved by investigations of oligomers containing these substituted nucleotides. Computational studies are another avenue that may aid in clarification the phenomena that have been presented. Investigations in different solvents may provide a better picture of the influence of surroundings on the properties of these interesting molecules.

The desirable emission wavelengths of the sulfur skeleton could be combined with the brightness of a chlorine substitution to make a bright analog with a large Stokes shift. Substituents tested thus far did not result in a large change in Stokes shift but further elaborations of these analogs or installation of additional functionality may make a useful pair of analogs for FRET studies with intrinsic nucleic acid probes. Although the synthetic route to the tricyclic tC⊙ is low yielding, it is possible that modification of the reaction conditions could provide access to this compound.

Further synthetic modification of compounds discussed in this work would be desirable. The OH-tC(o) analogs can be investigated on their own, or following further reaction with other new substituents via activation through the triflate. Buchwald Hartwig amination of the Cl-tC⊙ analogs are still a potential avenue to new fluorophores. Synthesis of Et₂N-tC analogs appears to have great potential through the thiazole chemistry. Synthesis of CF₃-tC(o) analogs may be the next great fluorescent nucleosides. Reactions of the substituents that are present will impart new functionality and provide additional library members to expand the present knowledge on substituent effects.
High performance liquid chromatography improvements in method development would likely increase the recovery of compound from the purification of these fluorescent nucleotides. Purification methods that are effective for the dimethoxytritylated forms of the nucleosides would also improve the advancement of these compounds towards future oligomer synthesis. Low reactivity may be circumvented through the use of more reactive starting materials. Phosphoramidite chemistry works well and purification conditions have been optimized for this synthetic step which follows the problematic protection of the 5’ position.

Synthesis from a nucleobase approach would be useful for the production of large amounts of nucleosides, as these reactants are tolerant to increases in scale. Finding the right protecting group could make all the difference in the synthesis of tC⁰s by these means. Similarly, the right protecting groups could make the hydroxy-cytosine a useful starting material for synthesis of new analogs.

The future of the tC(o) analog library is bright. Fluorescent nucleoside analogs are very useful in the study of bimolecular processes, and the technology is always improving. Single molecule studies are now possible, and would be greatly aided by brighter probes that are incorporated directly at the site of study.¹⁵⁵

Cl-tC⁰ nucleoside analogs have been synthesized which are more brilliant than the bright parent compounds. These bright analogs may have an environmental reporting ability that is missing from tC. Triphosphate versions of these compounds have been sent for enzymatic testing which could produce oligonucleotides for nucleic acid studies of these molecules. Investigations comparing these closely related analogs have provided
information that suggests relationships between photophysical properties and positions of substituents. This information will be useful in the design of new fluors with a specific property in mind. Synthetic routes to new substituents and related molecules have been presented and the toolbox for biophysical investigation of nucleosides and nucleic acids will continue to grow.
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