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Design and Synthesis of Novel Fluorescent Nucleoside Analogues

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Abstract

This project aimed to obtain a set of fluorescent nucleoside analogues that closely resemble cytosine with extending the aromatic surface (extended conjugation) for enhance the photophysical properties. In addition to synthesize new fluorescent nucleoside analogues and evaluate their photophysical properties, this project aimed to understand the effects of donor substituent on the tC photophysical properties.

The main goal of these studies is to develop highly fluorescent nucleotide analogues. We first were looking to synthesize tC fluorescence nucleotide with the pyrimido[4,5-b]quinolone. In this part, 2-chloro-3-formylquinoline was easily prepared, yet the cyclization of 2-chloro-3-formylquinoline with urea or guanidine HCl was the most challenging that faced us. However, we have synthesized 2-amino [4,5-b]quinoline-one ring tC fluorescence under super dry reaction conditions in <10% yield.

The second goal was synthesizing new modified fluorescent nucleoside derivatives of tricyclic cytidine, which structurally mimics natural cytidine, and evaluates the photophysical properties of the new fluorescent tC nucleoside to understand the affects of the donor substituent, OCH₃, group on tC. In this part, we have reported new tC nucleoside derivatives with
electrons donor substituent OCH$_3$ group at carbon 7, tC$_{7OMe}$, and the other at carbon 8, tC$_{8OMe}$. These different substitution positions offer important distinctions between the design of the tC, tC$_{8OMe}$, and tC$_{7OMe}$. The unsubstituted tC nucleoside was easy to synthesize. In contrast, the synthetic strategy of the tC$_{7OMe}$ nucleoside that has electron donor substituent group OCH$_3$ located at carbon 7 and \textit{para} to amino group is synthetically less challenging and higher yielding than synthesis of tC$_{8OMe}$ which has OCH$_3$ group substituent \textit{meta} to amine group. Moreover, the photophysical properties of the fluorescent nucleoside derivatives of tC have been evaluated. Fluorescence studies of modified tC nucleosides demonstrated distinguish fluorescence emission and absorption wavelengths for tC$_{7OMe}$, and tC$_{8OMe}$, which displayed higher quantum yields and brightness compared to unmodified tC, dependent on mismatch position of the electron donor group (7 or 8) on the tC. This can be explained by the fact that the donor group OCH$_3$ which allow for extended conjugation of unshared pairs of electrons lead to increases the freedom of the $\pi$-electrons and likely to raise the fluorescence. Also, our fluorescence study established that there are effects of the different positions of substituent donor group. The fluorescence emission wavelength of tC$_{8OMe}$, where the OCH$_3$ is \textit{meta} to amino group, is red sifted longer than the fluorescence emission wavelength of tC$_{7OMe}$, where the OCH$_3$ is \textit{para} to amino group; however, tC$_{7OMe}$ exhibited higher quantum yield and brightness than tC$_{8OMe}$. 
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# Table of Contents

Abstract ................................................................................................................................. ii  
Acknowledgments ................................................................................................................ iv  
Table of Contents .................................................................................................................. v  
List of Figures ......................................................................................................................... vii  
List of Schemes ..................................................................................................................... ix  
List of Tables ......................................................................................................................... xi  
Abbreviations ......................................................................................................................... xii  

## Chapter One: Introduction

1.1. Deoxyribonucleotides (DNA) ...................................................................................... 1  
1.1.1. Major structures of DNA ...................................................................................... 3  
1.1.2. Nucleosides and Nucleotides ............................................................................... 4  
1.2. Fluorescence .............................................................................................................. 6  
1.2.1. The Fluorescence Process (Fluorescence Fundamentals) ............................... 6  
1.2.2. Fluorescence Spectroscopy ............................................................................... 7  
1.2.3. Fluorescence Resonance Energy Transfer (FRET) ........................................ 8  
1.3. Fluorescent Nucleotide Analogues ........................................................................... 11  
1.3.1. Classify the Fluorescent nucleobases ................................................................. 12  
   I. According to their Structure ..................................................................................... 12  
   II. According to their Environment-sensitive Analogues ........................................... 14  
1.4. Aims of the project .................................................................................................... 18  
   Hypothesis ................................................................................................................. 21  
1.5. References ............................................................................................................... 24

## Chapter Two: Synthesis of nucleobase analogues with the Pyrimido[4,5-b]quinoline-2(1H)-one ring system tC^c

2.1. Introduction .................................................................................................................. 27  
2.2. General Synthetic Elaborations ................................................................................. 29  
   2.2.1. Synthesis of tC_{COMe} nucleobase ................................................................... 30  
   2.2.2. Synthesis of tC_{Cex} nucleobase ...................................................................... 32
2.2.3. Synthesis of tC$^{\text{COH}}$ nucleobase ........................................... 34
2.2.4. Synthesis of tC$^{C}$ nucleobase ........................................... 35
2.3. Experimental ........................................................................ 37
2.4. Conclusion .......................................................................... 47
2.5. References ........................................................................... 49

Chapter Three: Synthesis and photophysical characterization of tC nucleoside derivatives

3.1. Introduction ........................................................................... 50
3.2. General Synthetic Elaborations ................................................. 53
  3.2.1. Synthetic of tC nucleoside ................................................. 54
  3.2.2. Synthetic of tC$_{\text{8OMe}}$ nucleoside ..................................... 58
  3.2.3. Synthetic of tC$_{\text{7OMe}}$ nucleoside ..................................... 62
  3.2.4. Synthetic of tC$_{\text{nitro}}$ nucleobase ..................................... 66
3.6. Photophysical properties of the fluorescent nucleosides .......... 70
  3.6.1. The Absorption of tC, tC$_{\text{8OMe}}$, tC$_{\text{7OMe}}$ ..................... 70
  3.6.2. Fluorescence Emission and Excitation of tC derivatives .......... 71
  3.6.3. Quantum yields ................................................................. 73
  3.6.4. Molar Extinction Coefficients ............................................. 75
3.7. Experimental ......................................................................... 77
3.8. Conclusion ........................................................................... 98
3.9. References ........................................................................... 102

Appendices

Appendix A .................................................................................. 103
List of Figures

Figure 1.1: Double-helix Structure of the DNA Molecule ........................................2
Figure 1.2: Watson-Crick base pairing between A:T and G:C ........................3
Figure 1.3: The structure of nucleosides and nucleotides....................................5
Figure 1.4: The purine nitrogenous bases derivatives ...........................................5
Figure 1.5: Fluorescent purine analogs .................................................................13
Figure 1.6: Fluorescent pyrimidine analogs ............................................................13
Figure 1.7: Fluorescent base analogues the pyrrolo-dC-'family .........................15
Figure 1.8: Fluorescent cytosine analogues developed by Sekine ....................16
Figure 1.9: BDF nucleoside analogues by Saito ..................................................16
Figure 1.10: Fluorescent base analogues Environment-sensitive ......................17
Figure 1.11: Tricyclic cytosine analogues: tC,tC\textsuperscript{O}, and tC\textsubscript{nitro} ....18
Figure 1.12: Fluorescent nucleoside analogues ....................................................19
Figure 1.13: Fluorescent tethered covalently to the base ..................................20
Figure 1.14: Fluorescent nucleoside analogues tC, tC\textsubscript{8OMe}, tC\textsubscript{7OMe} ....23
Figure 2.1: The similarity between tC\textsuperscript{c} and Cytosine ..........................28
Figure 2.2.1: The fluorescent tC\textsubscript{COMe} nucleobase ..................................30
Figure 2.2.2: The fluorescent tC\textsubscript{Cex} nucleobase ........................................32
Figure 2.2.3: The fluorescent tC\textsubscript{COH} nucleobase ...................................34
Figure 2.2.4: The fluorescent tC\textsuperscript{C} nucleobase .......................................35
Figure 3.1.1: tC, tC\textsubscript{8OMe}, tC\textsubscript{7OMe}, tC\textsubscript{nitro} ..................................52
Figure 3.1.2: Illustrations of the positions 7C and 8C ........................................53
Figure 3.2.1: The fluorescent tC nucleoside .......................................................54
Figure 3.2.2: The fluorescent tC\textsubscript{8OMe} nucleoside ......................................58
Figure 3.2.3: The fluorescent tC\textsubscript{7OMe} nucleoside ....................................62
Figure 3.2.4: The tC\textsubscript{nitro} nucleobase ......................................................66
Figure 3.6.1: The absorption spectra of tCs in EtOH and in H\textsubscript{2}O .............71
Figure 3.6.2a: The excitation and emission spectra of tC nucleosides derivatives in Ethanol.................................................................72
Figure 3.6.2b: The excitation and emission spectra of tC nucleosides derivatives in H$_2$O ................................................................. 73

Figure 3.6.3a: Plot of emission versus absorption .................................. 74

Figure 3.6.3b: Plot of integrated emission versus absorption to determine the quantum yield of tC$_{8OMe}$ ........................................................................ 75

Figure 3.6.3c: Plot of integrated emission versus absorption to determine the quantum yield of tC$_{7OMe}$ ................................................................. 75

Figure 3.6.4: Plot of absorption versus concentration .................................. 76
List of Schemes

Scheme 1.1: Diagram of Electronic Transitions ........................................... 7
Scheme 1.2: Diagram illustrating the FRET process ................................. 9
Scheme 1.3: Overview of energy transfer and fluorescence FRET ............... 10
Scheme 2.1: The expected tautomerism for hydroxy-tC^C .................................. 29
Scheme 2.2: General Synthetic Route of tC^C ........................................... 30
Scheme 2.2.1: The attempted synthetic pathway to tC^{COME} ...................... 31
Scheme 2.2.2: The attempted synthetic pathway to tC^{Cex} ....................... 33
Scheme 2.2.3: The attempted synthetic pathway to tC^{COH} ....................... 34
Scheme 2.2.4: The attempted synthetic pathway to tC^C .......................... 36
Scheme 2.3.1: The synthesis of 3'-methoxy acetonilide ................................. 38
Scheme 2.3.2: The synthesis of 2-chloro-7-methoxy-quinoline-3-carbaldehyde ................................................................................................. 39
Scheme 2.3.3: The attempted synthesis of tC^{COME} base .......................... 40
Scheme 2.3.4: The synthesis of 2-naphthylacetamide .................................. 41
Scheme 2.3.5: The attempted synthesis of tC^{Cex} ...................................... 42
Scheme 2.3.6: The attempted synthesis of tC^{Cex} ...................................... 43
Scheme 2.3.7: The synthesis of acetamide with protected group .................. 44
Scheme 2.3.8: The attempted synthesis of 2-chloro-3-formylquinoline protected ................................................................................................. 45
Scheme 2.3.9: The synthesis of 2-chloro-3-formylquinoline .......................... 46
Scheme 2.3.10: The synthesis of 2-amino-tC^c ........................................... 47
Scheme 3.2: Synthetic Elaboration of Fluorescent nucleoside tC ............... 54
Scheme 3.2.1: Synthetic pathway to tC nucleoside ..................................... 57
Scheme 3.2.2: Synthetic pathway to tC^{8OMe} nucleoside .......................... 61
Scheme 3.2.3: Synthetic pathway to tC^{7OMe} nucleoside .......................... 65
Scheme 3.2.4: Synthetic pathway to tC^{nitro} nucleobase ............................ 69
Scheme 3.7.1: The synthesis of 2,4-Dihydroxy-5-(2-amino-phenylthio) pyrimidine ................................................................................................. 78
Scheme 3.7.2: Synthesis of tC base ............................................................. 79
Scheme 3.7.3: Synthesis of 2-deoxy-3,5-di-O-p-toluoyl-phenothiazine .... 80
Scheme 3.7.4: Synthesis of tC nucleoside ....................................................... 81
Scheme 3.7.5: The synthesis of 4-methoxy-2-aminothiophenol .............. 83
Scheme 3.7.6: The synthesis of 2,4-Dihydroxy-5-(2-amino-4-methoxy phenylthio) pyrimidine ................................................................. 84
Scheme 3.7.7: The synthesis of 2′-deoxy tC_{8OMe} base ......................... 85
Scheme 3.7.8: The synthesis of 2′-deoxy-3′,5′-bis-O-p-toluoyl-8-methoxyphenothiazine ................................................................. 86
Scheme 3.7.9: The synthesis of 3-(2-Deoxy-b-D-ribofuranosyl)-8-methoxy-1,3-diaza oxo-phenothiazine ........................................ 88
Scheme 3.7.10: The synthesis of 1-(5-methoxy-2-aminothiophenol)-5-methoxy-2-aminothiophenol ............................................................. 90
Scheme 3.7.11: The synthesis of 2,4-Dihydroxy-5-(2-amino-5-methoxy phenylthio) pyrimidine ................................................................. 91
Scheme 3.7.12: The synthesis of tC_{7OMe} base ........................................ 92
Scheme 3.7.13: The synthesis of 2′-deoxy-3′,5′-bis-O-p-toluoyl-7-methoxyphenothiazine ................................................................. 93
Scheme 3.7.14: The synthesis of 3-(2-Deoxy-b-D-ribofuranosyl)-7-methoxy-1,3-diaza oxo-phenothiazine ................................................... 94
Scheme 3.7.15: The synthesis of 2,4-Dihydroxy-5-(2-amino-5-nitrophenylthio) pyrimidine ................................................................. 96
Scheme 3.7.16: The synthesis of tC_{nitro} base .......................................... 97
List of Tables

Table 3.6.1: Absorption and Fluorescence emission maxima of tC derivatives in H₂O and in EtOH ........................................ 73

Table 3.6.2: Fluorescence quantum yields, molar absorptivity, and brightness in EtOH .................................................. 77
List of Abbreviations

A        adenine
BDF      base-discriminating fluorophore
°C       degrees Celcius
C        Cytosine
Calcd.   Calculated
δ        Chemical shift
DMF      dimethylformamide
DMSO     dimethylsulfoxide
DMT      4,4’-dimethoxytrityl
DMTCl    Dimethoxytrityl chloride
DNA      deoxyribonucleic acid
EtOH     ethanol
FRET     Fluorescence resonance energy transfer
g        gram
G        guanine
h        hour
MS       Mass spectrometry
µ        micro
MeOH     methanol
min      minutes
mL       milliliter
mmol     millimole
nm       nanometers
NEt3     triethylamine
NMR      nuclear magnetic resonance
NaOMe    Sodium methoxide
ppm      parts per million
PPh₃     triphenylphosphine
q        quartet
rt       room temperature
s  singlet
T  thymine
  triplet
THF  tetrahydrofuran
TLC  thin-layer chromatography
UV  ultraviolet
vis  visible
Φ_F  fluorescence quantum yield
VR  Vilsmeier's reagent
Chapter 1

Introduction

1.1 Deoxyribonucleotides (DNA)

In 1953 the three-dimensional structure of the DNA double helix, which is two polynucleotide chains twisted around each other bonded via hydrogen bonds in the form of a double helix (Figure 1.1), was discovered by Watson and Crick.\(^1\) Since that time, DNA, which is considered a key component of life, became the focus of many researchers. DNA (genetic material) \(^2\) is a molecule that is responsible for storage, duplication, and investigation of the genetic information in bodies of living organisms; therefore, it plays a vital role in all living organisms.\(^3\)
Figure 1.1: Double-helix Structure of the DNA Molecule
### 1.1.1 Major Structures of DNA:

In the double helix DNA the residues (nucleotides) are built up of four bases: adenine (A), thymine (T), cytosine (C) and guanine (G) that are connected via specific binding between the nitrogenous nucleobases known as Watson-Crick hydrogen bonding, adenine (A) always pairs with thymine (T) / (uracil (U) in case of RNA) and *visa versa*, while guanine (G) always pairs with cytosine (C) and *visa versa* \(^3\) (Figure 1.2).

![Figure 1.2: Watson-Crick base pairing between A: T and G:C](image-url)
1.1.2 Nucleosides and Nucleotides

Nucleotides are monomeric biomolecular units of nucleic acids (DNA and RNA) that are created from nucleosides. Nucleotides consist of three components covalently bonded together: \(^1\) (Figure 1.3)

I. Nitrogenous Bases (Heterocyclic bases)

The nitrogenous bases of DNA are divided into two classes

Purines (bicyclic)

1. Guanine. 2-amino-6-oxypurine (figure 1.4 a)
2. Adenine 6-aminopurine (figure 1.4 b)

Pyrimidines (monocyclic)

1. Cytosine 2-oxy-4-amino pyrimidine (figure 1.4c)
2. Thymine 2,4-oxy 5-methyl pyrimidine (DNA) (figure 1.4d)
3. Uracil 2,4-oxypyrimidine (RNA) (figure 1.4e)

II. A Pentose (5-carbon sugar) – ribose or 2'-deoxyribose.

These nitrogenous bases are connected to the five membered ring sugar, a furanose, via a \(\beta\)-glycosidic bond (figure 1.3).

III. A Phosphate Group

Nucleotides have one, two or three phosphate group(s), while the nucleosides have a hydrogen atom instead of the phosphate group(s).\(^1\)
Phosphate groups are bridge linkages that link nucleotides in a phosphodiester backbone between the 3' carbon of one deoxyribose molecule and the 5' carbon of another.

**Figure 1.3:** The structure of nucleosides and nucleotides

**Figure 1.4:** The purine nitrogenous bases derivatives (a) Guanine (b) Adenine

The pyrimidines nitrogenous bases derivatives (c) Cytosine (d) Thymine (e) Uracil.

R= H, ribose
1.2 Fluorescence:

1.2.1 The Fluorescence Process (Fluorescence Fundamentals)

The process of fluorescence can be illustrated in a simplified diagram known as the diagram of Electronic Transitions or Jablonski diagram (Scheme 1.1). Jablonski diagram shows that fluorescence is a result of two stages. The first stage is excitation which occurs when an electron is excited from the ground state ($S_0$) to a singlet lowest excited state ($S_1$) by absorption of light (photons). The second stage is relaxation from the excited state ($S_1$), via different processes involved: Fluorescence occurs when an electron relaxes from the lowest level of an excited singlet state ($S_1$) to the singlet ground state ($S_0$). Intersystem crossing occurs when a photon relaxes from the lowest level of an excited singlet state ($S_1$) to the triplet excited state ($T_1$). Phosphorescence occurs when a photon is released from the triplet excited state to the singlet ground state ($S_0$).
1.2.2 Fluorescence Spectroscopy

Fluorescence spectroscopy techniques are methods of analysis that depend on the fundamental nature of the transitions involved where the electrons of the analyte are excited to higher electronic states by irradiation (absorbed photons) at a certain wavelength and emit radiation of a different wavelength when relaxing back to the ground state.\(^6\)

Fluorescence spectroscopy techniques have features such as providing high detection sensitivity, unparalleled time resolution, and can easily be performed under several solution conditions.\(^7\) These characteristics make them classified as indispensable tools in the study of

---

**Scheme 1.1:** Diagram of Electronic Transitions (Jablonski Diagram)
structure and dynamics of biological molecules and study photophysical properties of fluorescent molecules, fluorophore (fluorochrome), such as absorption wavelength $\lambda_{\text{abs}}$, emission wavelength $\lambda_{\text{em}}$, quantum efficiency $\varphi_F$, and excited state lifetime $\tau$. Each fluorophore has a characteristic light excitation and emission spectrum where the emission energy is at lower energy and longer wavelength than the excitation energy.

1.2.3 Fluorescence Resonance Energy Transfer (FRET):

Förster resonance energy transfer, more commonly known as Fluorescence resonance energy transfer (FRET), is one of the most important fluorescence techniques that are utilized widely in biotechnology measurements, such as single-molecule imaging techniques, probes for structural and functional analyses, for investigating structure and dynamics of biopolymers, and molecular beacons.

FRET is not sensitive to the environment, as a result, the information of fluorescence measurements relate exclusively to the changes in the conformation of the biopolymer under study.
FRET mechanism (Figure 1.5), involves two fluorophores, a donor and an acceptor. Excitation of the donor by absorption of light at one wavelength results in transfer of energy directly to the acceptor which emits a radiation at a longer wavelength (Scheme 1.2). This process depends on certain criteria and requirements that must be met for FRET to occur. The physical orientation of the transition dipoles of donor and acceptor fluorophores must be approximately parallel to each other, and the distance between a donor and acceptor molecule. The effective distance between the donor and acceptor is in the range of 10–100 Å. In addition, the fluorescence emission spectrum of the donor and the absorption or excitation spectrum of the acceptor chromophore must overlap.\(^\text{10}^\)
Scheme 1.5: Overview of energy transfer and fluorescence FRET
1.3 Fluorescent Nucleotide Analogues

The normal nucleic acid bases provide short decay times (lifetime); as a result, the natural fluorescence of DNA or RNA is very weak;\textsuperscript{27} also the signals are effectively averaged over all the bases in the DNA chain; consequently, the natural nucleobases do not provide much structural information. The replacement of a normal base with a modified base can enhance the emissive properties of the nucleoside.\textsuperscript{7}

Many scientists are interested in synthesizing novel fluorescent nucleotide base analogues because of the importance of their utility in many analytical applications such as detection, quantification of nucleic acids and study of their structure, dynamics, hybridization, and interaction with other molecules.\textsuperscript{11} Therefore, fluorescent nucleic acid base analogues are very important probes in several fields such as organic chemistry, physics, biochemistry, and biology.\textsuperscript{11} Replacing the DNA bases by fluorescent hydrocarbons and heterocyclic nucleic acid base analogues helps to predict the location of the molecules.\textsuperscript{11}
1.3.1 Classification of Fluorescent nucleobases

I. According to Structure

Fluorescent nucleobases that resemble the natural DNA bases have several features that make them an important aim for researchers. That nucleobases which mimic the natural DNA nucleosides are classified into two groups according to their structure:

(a) Fluorescent purine analogues, such as 2-aminopurine (Figure 1.5a), Etheno-A (Figure 1.5b) 3-methylisoxanthopterin (Figure 1.5c), and 6-methylisoxanthopterine (Figure 1.5d)

(b) Fluorescent pyrimidine analogues, such as furyl-U (Figure 1.6a), pyrrolo-C (Figure 1.6b), 5-benzopyridopyrimidine(Figure 1.6c), and 3,5-diaza-4-oxophenothiazine (tC) (Figure 1.6d), bicyclic 4-N-carbamoyldeoxycytidine derivative, pyrrolopyrimidopyrimidine, and pyrimidopyrimidoindole nucleoside (dCPPI) (figure 1.8).
Figure 1.5: Fluorescent purine analogs (a) 2-Aminopurine (2-AP), (b) Etheno-A, (c) 3-MI and 6-MI, (d) 6-MAP and DMAP. R= (deoxy)ribose

Figure 1.6: Fluorescent pyrimidine analogs (a) 5-furyl-U, (2-AP), (b) pyrrolo-C, (c) 5-benzopyridopyrimidine, (d) 3,5-diaza-4-oxopenthiazine (TC). R= (deoxy)ribose
II. According to their Environmental Sensitivity

II.I Environmentally Sensitive Fluorescent Base Analogue s

Whereas fluorescent nucleobases have great utility as reporters for enzyme function and as probes of nucleic acid sequence and structure, some of the fluorescent nucleobases are environmentally sensitive and influenced by neighboring bases. Although several environmentally sensitive nucleobases have distinctive fluorescent properties and are used as powerful tools for investigating the perturbations, nucleic acid structure, dynamics, and recognition, they have some limitations. For instance, the environment-sensitive fluorescent base analogues could be affected by neighboring bases and lose their fluorescence, being quenched, during duplex formation probe. In addition to quenching by neighboring bases they usually produce low quantum yields. These probes can be categorized as: isomorphic base analogues, e.g. 2-aminopurine (2AP), pteridines e.g 3-methylisoxanthopterin (3-MI) and 6-methylisoxanthopterin (figure.1.5), and expanded nucleobases, e.g. pyrrolo-dc (figure.1.7) and some fluorescent cytosine analogues developed by Sekine et al (Figure 1.8) and BDF nucleoside analogues developed by Saito et al. (Figure 1.9) analogues
(a) 5-methylpyrimidin-2-one, (b) 7-deazapurines, (c) 5-alkynyluridines, (d) benzoquinazolines, (e) triazoleadenosines and (f) 1,6-ethenoadenosine

(Figure 1.10)\textsuperscript{16}

\textbf{Figure 1.7:} Fluorescent base analogues in the pyrrolo-dC\textsuperscript{*} family \textsuperscript{*}: (a) dF\textsuperscript{*}, (b) dF, (c) furano-dT and (d) pyrrolo-dC. R=(deoxy)nbose. Phenyl derivatives of pyrrolo-dC: (e) PhpC, (f) moPhpC and (g) boPhpC. R=(deoxy)nbose
**Figure 1.8:** Fluorescent cytosine analogues developed by Sekine et al.: (a) dC\(^{\text{PPP}}\), (b) dC\(^{\text{PP}}\) and (c) dC\(^{\text{PP}}\). \(R=\text{(deoxy)ribose}, R^1=\text{H, OMe, SMe}\)

**Figure 1.9:** BDF nucleoside analogues by Saito et al.: (a) BPP, (b) NPP, (c) \(\text{MDA}\), (d) \(\text{MDL}\) and (e) \(\text{NDA}\). \(R=\text{(deoxy) ribose}\).
II. II Environmentally Insensitive Fluorescent Base Analogues

Environment-insensitive fluorescent base analogues are the most important in experiments that depend on the emission for detection and where FRET and anisotropy measurements are used because they have shown a high fluorescent quantum yield when incorporated into oligonucleotides. Almost all fluorescent nucleobase analogs are quenched in duplex nucleic acids, whereas it has been demonstrated that the tricyclic cytosine analogue tC family, phenothiazine, 1,3-diaza-2-oxophenothiazine,
and tC\textsuperscript{O}, phenoxazine, 1,3-diaza-2-oxophenoxazine (Figure 1.11 a,b) are fluorescent have shown a high fluorescent quantum yield when incorporated into oligonucleotides. \textsuperscript{18} Also, the non-fluorescent tC\textsubscript{nitro} (Figure 1.11 c) is part of tC family consider as an important molecule because it is the first nucleobase FRET-pair, yielding accurate distance and orientation information in DNA. \textsuperscript{19,25,26}

![Figure 1.11: Tricyclic cytosine analogues: (a) tC, (b) tC\textsuperscript{O} and (c) base analogue FRET acceptor non Fluorescence tC\textsubscript{nitro} R= (deoxy) ribose](image)

### 1.4 Aims of the Project:

The goals of this project are: to synthesize novel fluorescent nucleoside analogues that can participate in Watson-Crick hydrogen bonding (Figure 1.12) and afford high brightness, quantum yields (\(\Phi_F\)), high molar extinction coefficient (\(\varepsilon\)), and distinguish absorptions. Also, to synthesize different derivatives of tC for understanding the affects of donor ring substitution at different positions.
Most fluorescent probes that exist today have limitations; for instance, some probes are very sensitive to the micro-environment when incorporated into an oligonucleotide and will be quenched, as it is mentioned previously. Also, some fluorescent nucleotides that currently exist have a fluorophore covalently attached via a flexible linker at C-5 eg. Cy5-dC, Cy3-dC (Figure 1.13), this flexibility of the linker could displace the fluorophore from the target position. Moreover, the flexibility impairs the use of the dye anisotropy, which prevented the direct observation of conformational rigidity imposed on the nucleobase. The fluorophore tethered bases have a different size and hydrophobicity compared to the natural base, thereby precluding their normal interaction with the DNA and RNA in the cell. Although Cy5 exhibit high red shift excitation at 635 nm and
emission at 670–720 nm \(^{22}\) with high quantum yield when linked to DNA, they are sensitive to their environment and it is worth mentioning that Cy3 fluorescence is sensitive to not only the closest base, but also to several additional bases farther away.\(^{23}\)

![Figure 1.13: Fluorescent base analogues consist of conventional dyes tethered covalently to the base (e.g. (a) Cy3-dC, (b) Cy5-dC).](image)

The tricyclic fluorescent base analogues tC and tC\(^O\) (figure 1.11 a,b) have been reported with unique fluorescence properties; for example, the tC derivatives are relatively insensitive to neighboring bases because their fluorescent intensity is virtually not affected by neighbors when it is incorporated into single and double-stranded oligonucleotides.\(^{12}\) In addition, they usually generate brightness and quantum yield tC\(^O\) is around 0.14 –
0.41, tC 0.2\textsuperscript{19} insensitive when incorporated into single and double-stranded oligonucleotides.\textsuperscript{12} Furthermore, these analogs are able to enhance the stability of oligodeoxynucleotides /RNA duplexes;\textsuperscript{18} thus, tC can be useful for investigating intrinsic characteristics of nucleic acid as well as interactions between nucleic acids and other molecules.\textsuperscript{16} Lin, K. et al. 1995, have found that both the phenothiazine and phenoxazine (Figure1.11) result in greatest duplex stability when tricyclic heterocycles are positioned together, showing π-π overlap between adjacent aromatic faces which is not possible in analogs of less than three rings.\textsuperscript{18} However, all base-pairing partners of tC that have been reported such as tC and tC\textsuperscript{O} (Figure 1.11) have similar absorption and emission wavelengths, so that it is difficult to distinguish between them. Therefore, we were looking forward to synthesizing novel modified tC fluorescent nucleoside analogs have higher brightness, less environmental sensitivity, and have distinguishable fluorescence emission and absorption wavelengths.

**Hypothesis**

We have assumed that extended the aromatic surface (extend the π system) of the natural base, such as the pyrimidine, results in useful
fluorescent properties. Accordingly, we were looking for synthesizing pyrimido[4,5-b]quinoline-2(1H)-one ring system tC\textsuperscript{C} derivatives.

Furthermore, according to the fact that photophysical characteristics (e.g., red shifted absorption and high emission quantum efficiencies) depend on the structure of the molecule.\textsuperscript{20} We expected that the functional groups which allow for extended conjugation of unshared pairs of electrons, such as alcohol, amine, amide, and ether, would raise the fluorescence intensity and the quantum yield. Moreover, we have hypothesized that the position of the substituent would affect the fluorescence intensity, the absorption, and emission wavelength, and the quantum yield.\textsuperscript{21}

In order to understand the effects of the donor substituent group OCH\textsubscript{3}, we reported two new nucleosides, tC\textsubscript{8OMe} and tC\textsubscript{7OMe}, that arose from the design of the tC nucleosides as will discuss in Chapter 3. Although the tC\textsubscript{8OMe} and tC\textsubscript{7OMe} nucleosides arose from the skeleton of the tC nucleosides, the modification function groups, OCH\textsubscript{3}, at different positions 7C and 8C would bring different photophysical properties according to the electronic nature of substituent and the substituent position.
Figure 1.14: Fluorescent nucleoside analogues (1) tC, (2) tC8OMe, (3) tC70Me
1.5 References


Chapter 2

Nucleobase analogues with the pyrimido[4,5-b]quinoline-2(1H)-one ring system \( \text{tC} \)

2.1 Introduction

In this part of our procedure, the main goal was to develop highly fluorescent nucleotide analogues. We were looking to synthesize the \( \text{tC} \) fluorescent nucleotide with the pyrimido[4,5-b]quinoline-2(1H)-one ring system (Figure 2.1).

Pyrimidoquinolines derivatives are an important class of heterocyclic molecules because of their partial similarity to the natural acid bases that enhances the probability of being able to act as a nucleobase (Figure 2.1). In general, quinoline molecules have been utilizing extensively in medicinal chemistry and biomedical field.\(^1\)\(^2\)\(^6\) For instance, they are active as antiarrhythmic, antimalarials, antimicrobial, anti-inflammatory, anticancer,\(^1\) and analgesic agents.\(^3\) As a result of the wide ranging applications of quinoline derivatives, the synthesis of quinoline derivatives became an attractive goal for many scientists.
The intermediate compound 2-chloro-3-formylquinoline was easily prepared, yet the cyclization of 2-chloro-3-formylquinoline with urea or guanidine HCl was the most challenging aspect of the synthesis. However, we have successfully synthesized 2-amino [4,5-b]quinoline-one ring tC\textsuperscript{C} under super dry reaction conditions with <10 yield.

![Chemical Structure](image)

**Figure 2.1:** The similarity between tC\textsuperscript{C} and Cytosine

The tricyclic cytosine nucleobases allow for the formation of two tautomeric configurations. \textsuperscript{4} This tautomerization provides two possible configurations which are acceptor-acceptor-acceptor (AAA) and donor-acceptor-acceptor (DAA) (Scheme 2.1).
Scheme 2.1: The expected tautomeration of hydroxy-tC will allow for it to attain the C-like donor-acceptor-acceptor pattern for Watson-Crick hydrogen bonding.

2.2 Synthesis of tC nucleobase analogues

The reactions sequences were begun with acetanilides, which were easily converted to 2-chloro-3-formylquinolines by treatment with the Vilsmeier reagent. Unfortunately, condensation of 2-chloro-3-formylquinoline with urea in a heated solution of acetic acid was not successful in producing the fluorescent tricyclic tC analogues pyrimido[4,5-b]quinoline-2(1H)-one. Instead the compound 2-chloro-3-formylquinoline was hydrolyzed because of water present in the reaction mixture that acts as a nucleophile and causes substitution at the chlorine atom preventing coupling with urea (Scheme 2.2).
2.2.1 Synthesis of tC$^{COMe}$ nucleobase

In order to synthesize 8-methoxy-pyrimido[4,5-b]quinoline-2(1H)-one tC$^{C}$ (Figure 2.2.1), the first step was to prepare 3'-methoxy acetonilide (2) which was obtained by reacting acetic anhydride with m-anisidine in the presence of pyridine at room temperature. Subsequent synthesis of 2-
chloro-7-methoxy-quinoline-3-carbaldehyde (3) was completed by treatment of 3'-methoxy acetonilide (2) with dimethylformamide (DMF) and phosphoryl chloride (Vilsmeier’s reagent, VR).\(^7\) Unfortunately, cyclization of (3) with urea and acetic acid in order to generate the target product 8-methoxy-pyrimido[4,5-b]quinoline-2(1H)-one tC\(^C\) (4) did not occur probably because of water present in the sample that hydrolyzes the intermediate compound 2-chloro-3-formylquinoline preventing the coupling with urea. The scheme 2.2.1 illustrates the attempted synthetic pathway to tC\(^{COMe}\).

Scheme 2.2.1: The attempted synthetic pathway to tC\(^{COMe}\)
2.2.2 Synthesis of tC\textsuperscript{cex} nucleoside

![Figure 2.2.2: The fluorescent tC\textsuperscript{cex} nucleoside](image)

In order to prepare methoxy-tetracyclic cytosine tC\textsuperscript{cex} \textsuperscript{(9)} (Figure 2.2.2), the reaction started by preparing 2-naphthylacetamide \textsuperscript{(6)} which was obtained by reacting acetic anhydride with 2-naphthylamine \textsuperscript{(5)} in the presence of pyridine at room temperature. The attempt to prepare 2-chloro-3-formyl-2-naphthylquinoline \textsuperscript{(8)} by treatment of 2-naphthylacetamide \textsuperscript{(6)} with dimethylformamide (DMF) and phosphoryl chloride (Vilsmeier's reagent, VR) produced compound \textsuperscript{(7)} instead of \textsuperscript{(8)} because of the preferred reactivity of electrophiles at position 8 over the position 2 in quinolines. Unfortunately, condensation of \textsuperscript{(7)} with urea in a heated solution of acetic acid was not successful in producing the fluorescent tricyclic analogues methoxy-tetracyclic cytosine tC\textsuperscript{cex} \textsuperscript{(9)}. 
Scheme 2.2.2: The attempted synthetic pathway to tCex
2.2.3 Synthesis of tC$^{COH}$ nucleobase

![Figure 2.2.3: The fluorescent tC$^{COH}$ nucleobase]

In order to prepare the molecule tC$^{COH}$ (Figure 2.2.3) the reaction was started by preparing acetamide (10) with a protected substituent group. This was obtained by treatment of 3-acetamidophenol (9) with NaH and methyl chloromethyl ether (MOMCl) commonly used as a protecting group for alcohols. Treatment of the acetamide (10) with Vilsmeier's reagent was not successful in producing the produce (11).

![Scheme 2.2.3: The attempted synthetic pathway to tC$^{COH}$]

34
2.2.4 Synthesis of tC<sup>C</sup> nucleobase

![Chemical Structure](image)

**Figure 2.2.4:** The fluorescent tC<sup>C</sup> nucleobase

In order to prepare pyrimido[4,5-b]quinoline-2(1H)-one ring tC<sup>C</sup>, (16) (Figure 2.2.4) the reaction was started by preparing 2-chloro-3-formylquinoline (14) from the treatment of acetanilide (13) with dimethylformamide (DMF) and phosphoryl chloride (Vilsmeier's reagent, VR). We were able to cyclize the 2-chloro-3-formylquinoline (14) with guanidine HCl in the presence of sodium hydride base to generate the target derivative tC<sup>C</sup> product (16) under super dry conditions with < 10% yield.
Scheme 2.2.4: The attempted synthetic pathway to tC^C
2.3 Experimental

**Synthesis of nucleobase analogues with the pyrimido[4,5-b]quinoline-2(1H)-one ring system tC**

**General**

Solvents and reagents were obtained from commercial suppliers and used without further purification. All moisture and oxygen sensitive reactions were carried out under nitrogen. Air and moisture sensitive solvents and solutions of reactions were transferred using syringes. Reactions were monitored by thin layer chromatography (TLC). NMR spectra were recorded on Bruker Avance 500MHz spectrometer. Unless otherwise specified, purifications were performed using manual flash column chromatography.
3’-methoxy acetanilide (2).

*m*-anisiden (1) (44.5mmol, 5 mL) was added to pyridine (50 mL) in acetic anhydride (17 mL). The mixture was stirred at room temperature for 3 h. The reaction was quenched by addition of methanol. The crude product was dissolved in ethyl acetate and washed with 1M HCl (2 x 100 mL) then with NaHCO₃ (2 x 100 mL) then brine (1 x 100 mL). The organic layer was dried over anhydrous Na₂SO₄. The solvent was evaporated using a rotary evaporator. Purification via flash column chromatography on silica gel (6% CH₃OH in CH₂Cl₂) afforded the desired product (2) that was identified via ¹H NMR (6.54 g, 89% yield) (scheme 2.3.1). ¹H NMR (500 MHz, Chloroform-d, 294 K): δ 7.64 (1 H, s), 7.27 (1 H, s), 7.19 (1 H, t, J 8.1), 6.98 (1 H, dd, J 8.0, 1.9), 6.65 (1 H, dd, J 8.2, 2.5), 3.77 (3 H, s), 2.15 (3 H, s).
Scheme 2.3.2: The synthesis of 2-Chloro-7-methoxy-quinoline-3-carbaldehyde

2-chloro-7-methoxy-quinoline-3-carbaldehyde (3).

Dry DMF (5.75 mL) and POCl₃ (7.9 mL) were stirred at 0°C under nitrogen. 3'-methoxy-acetanilide (2) (30.26 mmol, 5 g) was added to the solution. The reaction mixture was stirred at room temperature 10 min then heated at 75°C for 4 h. The progress of the reaction was monitored by TLC. The liquid product was added to ice water generating yellow precipitate that was collected by filtration and washed with water yielding the desired product (3) (3.55 g, 53% yield) (scheme 2.3.2). ¹H NMR (500 MHz, DMSO-d₆, 294 K): δ 12.09 (1 H, s), 10.18 (1 H, s), 8.44 (1 H, s), 6.89 (1 H, d, J 8.8, 2.4), 6.84 (1 H, d, J 2.5), 3.86 (3 H, s).
Scheme 2.3.3: The attempted synthesis of tC<sub>COMe</sub> base

8-methoxy-pyrimido[4,5-b]quinoline-2(1H)-one tC<sup>C</sup> base (4).

2-chloro-7-methoxy-3-formylquinoline (3) (4.51 mmol, 1 g) was added to urea (47.95 mmol, 2.88 g) in acetic acid (20 mL). The mixture was heated at 80°C for 2 h. The liquid product was added to ice water and then sodium bicarbonate was added slowly producing orange precipitate that was collected by filtration and washed with water. Orange fluorescent powder was the obtained product. Unfortunately, the NMR measurement shows only the hydrolyzed compound probably because of water present in the reaction (scheme 2.3.3).
2-naphthylacetamide (6).

2-naphthylamine (5) (6.26 mmol, 0.9 g) was stirred in acetic anhydride (2.3 mL) in the presence of pyridine (7.1 mL) at room temperature for 3 h. Methanol was added to quench the reaction. The crude product was dissolved in ethyl acetate and washed with 1M HCl (2 x 100 mL) then with NaHCO$_3$ (2 x 100 mL) then brine (saturated NaCl 1x 100 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, and the solvent was removed by evaporation. Purification via flash column chromatography on silica gel (6% CH$_3$OH in CH$_2$Cl$_2$) afforded the desired product (6) (0.8296 g, 71.5% yield) (scheme 2.3.4). $^1$H NMR (500 MHz, Chloroform-d, 294 K): δ 8.19 (1 H, s), 7.77 (4 H, dd, J 7.3, 4.6), 7.45 (2 H, ddd, J 8.1, 4.2, 1.9), 7.39 (1 H, s), 2.23 (3 H, s).
2-chloro-3-formyl-naphthylquinoline (8).

Dry DMF (0.72 mL) and POCl$_3$ (2.5 mL) were stirred at 0°C under nitrogen. 2-naphthylacetamide (6) (4.427 mmol, 0.829 g) was added to the solution and the reaction mixture was stirred at room temperature for 10 min. Then the mixture was heated at 75°C overnight. Then the liquid product was added to ice water generating precipitate. The orange precipitate was collected by filtration and washed with water. Purification via flash column chromatography on silica gel (15% ethyl acetate in hexanes
then 30% ethyl acetate in CH$_2$Cl$_2$) resulted in the desired product (7) which was collected and concentrated by evaporating the solvent with a rotary evaporator (0.879 g, 72% yield) (scheme 2.3.5). $^1$H NMR (500 MHz, Chloroform-d, 294 K): δ 10.64 (1 H, s), 9.51 (1 H, s), 8.71 (1 H, d, J 8.3), 8.17 (1 H, d, J 9.1), 8.00 (1 H, d), 7.96 (1 H, d, J 9.2), 7.81 (1 H, t, J 8.0), 7.75 (1 H, t, J 7.5).

Scheme 2.3.6: The attempted synthesis of tc$^{C\text{ex}}$

Methoxy-tetracyclic cytosine tc$^{C\text{ex}}$ (9).

2-chloro-3-formyl naphthyl quinoline (7) (0.413 mmol, 0.1 g) was added to urea (0.413 mmol, 0.028) in (2 mL) acetic acid. The mixture was heated at 80° C 2 h. The liquid product was added to ice water and then sodium bicarbonate was added producing an orange precipitate that was collected.
by filtration and washed with water. Orange fluorescent powder was obtained (Scheme 2.3.6). Purification was performed via flash column chromatography on silica gel (4% methanol in CH₂Cl₂), but ¹H NMR (500 MHz, CDCl₃) measurements showed the target product tC_Cex was not produced.

![Scheme 2.3.7: The synthesis of acetamide with protected group](image)

acetamide with protected group (10).

3-acetamidophenol (9) (6.6 mmol, 1g) was added to NaH (7.25 mmol, 0.174 g) dissolved in THF (20.6 mL) at 0°C under nitrogen. After 3 h, MOMCl (0.5 mL) was added at 22°C overnight. Purification via flash column chromatography on silica gel (4% methanol in dichloromethane) resulted in the desired product (10) (1.098 g, 76.5% yield) (Scheme 2.3.7). ¹H NMR (500 MHz, Chloroform-d, 294 K): δ 8.35 (1 H, s), 7.15 (1 H, d, J 1.7), 7.14 (1 H, d, J 1.2), 6.74 – 6.70 (1 H, m), 5.26 (1 H, s), 5.11 (1 H, s), 3.42 (3 H, s), 2.11 (3 H, s).
protected 2-chloro-3-formylquinolines (11).

Dry DMF (1.14 mL) and POCl₃ (3.93 mL) were stirred at 0 °C under nitrogen. Acetanilide (10) (6.034 mmol, 1.178 g) was added to the solution and the reaction mixture was stirred at room temperature for 10 min. Then the mixture was heated at 75 °C for 4 h. The progress of the reaction was monitored by TLC. The liquid product was added to ice water and then sodium bicarbonate was added producing an orange precipitate that was collected by filtration and washed with water. Orange fluorescent powder was obtained. Purification was performed via flash column chromatography on silica gel (6% methanol in dichloromethane) but the ¹H NMR (500 MHz, CDCl₃) (Scheme 2.3.8) measurements showed the target product (11) was not produced.
2-chloro-3-formylquinoline (14).

Acetanilide (13) (24 mmol, 3.24 g) was added to a stirred solution of POCl₃ (16 mL) in dry dimethylformamide, DMF, (4.6 mL) at 0°C under nitrogen. After 10 min stirring at room temperature the mixture was heated at 75°C for 16h. The progress of the reaction was monitored by TLC. Then the liquid product was added to ice water and was neutralized by adding potassium carbonate generating yellow precipitate (14) in (2.94 g, 64% yield) (Scheme 2.3.9).
Scheme 2.3.10: Synthetic 2-amino-pyrimido[4,5-b]quinolin-one ring tC^C

2-amino-pyrimido[4,5-b]quinoline-one ring tC^C(16).

To a stirred solution of guanidine HCl (0.96 mmol, 0.09 g) in DMF (2 mL) over molecular sieves sodium hydride 60% in oil (0.64 mmol, 0.026 g) was added. After 60 min 2-chloro-3-formylquinoline (0.16 mmol, 0.03 g) (14) was added and the mixture was heated at 80^o C for 1 h. The precipitate produced was filtered and washed with 1:1 CH₃OH: CH₂Cl₂. The purification via flash column chromatography on silica gel (20% methanol in dichloromethane) afforded the desired product 2-amino-tC^C (16) (<10 % yield) (Scheme 2.3.10).

2.4 Conclusion:

Since there are published papers that show how easy the synthesis of tC^C fluorescent nucleotide is with the pyrimido[4,5-b]quinoline-2(1H)-one ring system,¹ we spent a long time attempting to prepare tC^C derivatives. We exactly followed some published procedures; however, using urea in
heated acetic acid for the cyclization reaction of 2-chloro-3-formylquinoline failed even under super dry conditions as the hydrolysis of 2-chloro-3-formylquinoline takes place instead of the cyclization. Fortunately, using guanidine HCl in the last reaction for the cyclization worked as the nucleophile is stronger than urea because of the unshared pair of electrons on the nitrogen atom which gives more of a chance for the cyclization to occur instead of hydrolysis. We have successfully synthesized 2-amino[4,5-b]quinoline-one ring under super dry reaction conditions with low yield <10%.
2.5 References


Chapter 3

Synthesis and photophysical characterization of tC nucleoside derivatives

3.1 Introduction

In this part of our procedure, we have designed two new fluorescent nucleosides analogues tC$_8$OMe, tC$_7$OMe (Figure 3.1.1); in addition we evaluated some of their photophysical properties as compared to Matteucci’s phenothiazine (tC). Moreover, we were looking for an improved synthetic route to prepare the non fluorescent nucleobase tC$_{nitro}$ which is considered to be an important molecule that uses as acceptor in FRET process.

As stated previously (in chapter 1), this project aimed to obtain a set of fluorescent nucleoside analogues that closely resemble cytosine by extending the aromatic surface (extended conjugation) to enhance the photophysical properties. In addition to synthesizing new fluorescent
nucleoside analogues and evaluating their photophysical properties, this project aimed to understand the effects of donor substituent on the photophysical properties of tC.

We are interested in synthesizing new fluorescent nucleoside tC derivatives because of the unique properties of the tC family. For example, the size and structure of tC analogues enable them to be incorporated site-specifically into nucleic acids with minimal disturbance of the native structure. Furthermore, tC analogues have shown their emission quantum and yield to be insensitive to changes in close bases after being incorporated into oligonucleotides. Consequently, tC is significantly more useful for distance determinations than the other available fluorescent base analogues which have sensitive emission and quantum yields. However, tricyclic cytosine tC analogues have exhibited relatively low quantum yields around 0.2 for the tC analogue and around 0.14 – 0.41 for tC. As a result, we were looking to improve the quantum yield by introducing tC derivatives which have a heteroatom-containing substituent such as a methoxy group.
These substituents allow for extended conjugation of unshared pairs of electrons leading to high fluorescence quantum yields, and red-shifts in emission spectra. Also, we have studied the effect of the position of the substituents.

![Chemical structures of nucleosides](image)

**Figure 3.1.1:** Fluorescent nucleoside analogues (1) tC, (2) tC$_{8OMe}$, (3) tC$_{7OMe}$, (4) tC$_{nitro}$

In order to provide novel structures that result in excellent photophysical properties for use as probes, we have designed modified novel nucleosides that are similar to the core structure of Matteucci’s phenothiazine (tC)$_2$ nucleoside with substituted electron donor group, OCH$_3$, which allows for extended conjugation of unshared pairs of electrons, at different positions 7C and 8C. These different substitution positions offer important distinctions between the design of the tC, tC$_{8OMe}$, and tC$_{7OMe}$. The tC nucleoside without any additional functional groups was easy to
synthesize. In contrast, the synthetic strategy for the tC<sub>7OMe</sub> nucleoside that has a functional group OCH<sub>3</sub>, substitution para to the amine group synthetically less challenging and higher yielding than synthesis of tC<sub>8OMe</sub> which has the OCH<sub>3</sub> group substitution meta to the amine group. However, the fluorescence emission wavelength of tC<sub>8OMe</sub> shifted farther than the fluorescence emission wavelength of both tC and tC<sub>7OMe</sub>.

![Figure 3.1.2: Illustrations of the positions of 7C and 8C](image)

**3.2 General Synthetic Elaborations:**

In general, the synthesis of fluorescent nucleobase analogues of the tricyclic cytosine (tC) family was convergent and was carried out in four main steps (scheme 3.2.1) (a) coupling, (b) ring-closure, (c) glycosylation, and (d) hydrolysis, which will be clearly explained for each synthesis.
Scheme 3.2: Synthetic Elaboration of Fluorescent nucleoside analogues tC
(a) coupling, (b) ring-closure, (c) glycosylation, (d) hydrolysis

R: H, OCH₃, or NO₂
X: S,O

3.2.1 Synthesis of tC nucleoside

Figure 3.2.1: The fluorescent tC nucleoside
The fluorescent tC nucleoside, Matteucci's phenothiazine, (Figure 3.2.1) was synthesized in four steps (Scheme 3.2.1). The intermediate (3) and tC base (4) were synthesized using the same pathway that has been used in an article which was published by Wilhelmsson and coworkers. Beginning with the combination of 5-bromouracil (2) with 2-aminothiophenol (1) heated in ethylene glycol in the presence of Na₂CO₃ for 1 h generated 62% yield (3). Followed by the ring-closure step that was started by heating the product (3) in a solution of EtOH and HCl for 22 h produced tC base (4) in 73% yield. For the glycosylation, using sodium hydride (60% in mineral oil), which is a strong base, deprotonated O-H giving an alkoxide anion (R-O⁻ Na⁺) salt, which has limited solubility in DMF. In this case, the glycosylation reaction has the chance to occur at the anion oxygen as well as at the nitrogen in position 2, the yield of 3-(2-Deoxy-b-D-ribofuranosyl)-1,3-diaza-2-oxophenothiazine was poor ~ 10–15%. We were able to increase the glycosylation yield to 40% for a mixture of β and α anomeric 2'-deoxy-3',5'-di-O-p-toluoyl-phenothiazine (5) by using the same pathway which was published in an article by Leconte. Using the Bis(trimethylsilyl)acetamide we were able to protect the oxygen on the tC base (silylation of alcohol) thus, the glycosylation reaction was directed
solely toward N-2 and the coupling was achieved in 40% yield. The separation of individual β and α anomers is poor at this step, thus the anomeric mixture was carried through to the next step. Toluoyl deprotection of the toluoylated phenothiazine (5) was achieved by using sodium methoxide in methanol for a combined yield of 0.0685 g, 43%. The free nucleosides were isolated separately β (6) and α (7) and identified depending on the elution profile from automated chromatography using an Isco gradient (CombiFlash® Rf ) and confirmed via $^1$H NMR 500.
Scheme 3.2.1: The synthetic pathway to tC nucleoside
3.2.2 Synthesis of tC$_{8OMe}$ nucleoside

Synthesis of the tC$_{8OMe}$ nucleoside (Figure 3.2.2) was carried out in six steps (Scheme 3.2.2). In order to prepare the starting material 2-amino-4-methoxythiophenol (10), the reaction was started with heating 5-methoxy-2-methylbenzothiazole ethylene glycol and NaOH under nitrogen gas. Unfortunately, the resulting mixture contained more disulfide (9) than the target product 2-amino-4-methoxythiophenol (10). However, purification and isolation of the disulfide (9) produced just ~ 5% yield pure material. So we changed the procedure in order to improve the yield of the disulfide (9) by adding H$_2$O$_2$. 1-(4-methoxy-2-aminothiophenol)-4-methoxy-2-aminothiophenol disulfide, (9) was successfully reduced to generate 2-amino-4-methoxythiophenol (10). Reduction was done by treating it with dithiothreitol, DTT, or triphenylphosphine, PPh$_3$. Using triphenylphosphine
produced triphenylphosphine oxide which is not easy to isolate from the target product, whereas using DTT enabled reduction of the disulfide bond and easy access to the intermediate 2-amino-4-methoxy-thiophenol (10). It may be useful to reduce the disulfide bond with PEt₃ which was useful in reducing the disulfide bone in tC₇OMe preparation (see below), but we did not try using PEt₃ with this molecule. By adding 5-Bromouracil and Na₂CO₃ into the previous mixture and heating at 120˚C, coupling was achieved, yielding product (11) in 33% yield. The attempted synthesis of tC₈OMe base (12) using the same pathway that was used in an article published by Wilhelmsson and coworkers which is, heating the product (11) in EtOH and HCl solution at 80˚C for 44 h produced product (12) in low yield ~ 5%. Fortunately, we have improved the yield to 30 % by changing the solvent to butanol instead of ethanol, thus we were able to heat the reaction at 116˚C for 20 h generating the tC₈OMe base (12) in 30% yield. For the glycosylation and for hydrolysis of toluoyl groups, we have used the same pathway that was previously used by Leconte. The tC₈OMe base (12) was coupled to 3’,5’-bis(toluoyl)-2-deoxyribosyl in the presence of Bis(trimethylsilyl)-acetamide to protect the oxygen on the tC₈OMe base (silylation of alcohol). Thus, the glycosylation reaction was directed only toward N-2 of compound
producing a mixture of β and α anomers of 2′-deoxy-3′,5′-di-O-p-toluoyl- 8-methoxy-phenothiazine (13) in 40% yield. The separation of β and α is poor at this step, thus the anomeric mixture was carried through to the next step. Toluoyl deprotection of the toluoylated 8-methoxy-phenothiazine (13) was achieved by using sodium methoxide in methanol, and the free nucleoside were isolated separately β (14) and α (15), identified depending on the elution profile from automated chromatography using Isco gradient (CombiFlash® Rf) (100:0 →60:40, CH₂Cl₂: MeOH) then confirmed via ¹H NMR 500. The β & α combined yield is 0.042 g, 34%. 

Scheme 3.2.2: The synthetic pathway to tC_{8OMe} nucleoside
3.2.3 Synthesis of tC<sub>7</sub>O<sub>Me</sub> Nucleoside

Synthesis of the tC<sub>7</sub>O<sub>Me</sub> nucleoside (Figure 3.2.3) was carried out in six steps (Scheme 3.2.3). In order to prepare the starting material 2-amino-5-methoxythiophenol (18), synthesis was first attempted using the same Wilhelmsson procedure using NaOH in ethylene glycol and heating at 120 °C, that had been used previously for preparing the tC<sub>8</sub>O<sub>Me</sub> base, but this produced a polymeric material, which we did not characterize. As a result, we changed the procedure by heating 6-methoxy-2-methylbenzothiazole in hydrazine monohydrate followed by oxidation of the sulfide molecule by the addition of hydrogen peroxide after cooling the reaction down to room temperature. The disulfide (17) was successfully reduced to generate the intermediate 2-amino-5-methoxythiophenol (18) by treating it with either triethylphosphine in 1M tetrahydrofuran, PEt<sub>3</sub>, or triphenylphosphine. Using
triphenylphosphine produced triphenylphosphine oxide which is not easy to isolate from the target product, whereas using PEt$_3$ we were able to reduce the disulfide bond easily and this gave access to the intermediate 2-amino-5-methoxythiophenol (18). By adding 5-Bromouracil and Na$_2$CO$_3$ into the previous mixture and heating at 120°C, coupling was achieved, yielding product (19) in 35% yield. First attempts to synthesize the tC$_7$OMe base (20) were made by using the same pathway that had been used previously for the tC$_8$OMe base by addition of HCl immediately to the mixture of starting material and butanol at room temperature then heating the mixture at 160°C. This procedure did not produce any reaction because the starting material had limited solubility. As a result, we changed the pathway by stirring compound (19) in butanol at 100°C for 10 min; in order to more completely the dissolve product (19) then added HCl after cooling the mixture down to room temperature for 5 min. The coupling was achieved by heating the reaction mixture at 116 °C overnight, an orange precipitate was generated which became yellow after washing with water yielding 61%. tC$_7$OMe base (20). No further purification was necessary for the tC$_7$OMe base (20) which has been confirmed by NMR).
For the glycosylation and for the hydrolysis of toluoyl groups, we have used the same pathway that previously was used by Leconte. The tC$_7$OMe base (20) was coupled to 3´,5´-bis(toluoyl)-2-deoxyribo-syl in the presence of Bis(trimethylsilyl)acetamide that was able to protect the oxygen on the tC$_7$OMe base (silylation of alcohol) thus, the glycosylation reaction was directed only toward the N-2 of compound (20) producing mixture of β and α anomers of 2´-deoxy-3´,5´-di-O-p-toluoyl-7-methoxy-phenothiazine (21) in 47% yield. The separation of β and α anomers is poor at this step, thus the anomeric mixture was carried through to the next step. Toluoyl deprotection of the toluoylated 7-methoxy-phenothiazine (21) was achieved by using sodium methoxide in methanol, and the free nucleoside anomers were isolated separately β (22) and α (23) were identified depending the elution profile from automated chromatography using an Isco gradient (CombiFlash® Rf 100:0 → 60:40, CH$_2$Cl$_2$: MeOH) then confirmed via $^1$H NMR 500 and MS analysis. The combined yield of β & α was 0.0057g, 67%.
Reducing agent: PPh₃ or Ph₃P

Acid: HCl or H₂SO₄

Solvent: n-butanol

**Scheme 3.2.3:** The synthetic pathway to TC₇0Me
3.2.4 Synthesis of tC_{nitro} Nucleobase

![Figure3.2.4: The tC_{nitro} nucleobase](image)

tC_{nitro} nucleobase (Figure 3.2.4) which was reported by Börjesson in 2009 as the first base analogue FRET acceptor \(^4\), was synthesized in four steps are shown in Scheme (3.2.4). Unfortunately, using the same pathway that has been used in an article that was published by Börjesson\(^4\) for the synthesis of this compound (27) generated a poor yield of around 12 %. This was the result of most of product oxidizing the starting material and thus producing a disulfide bond between two 5-nitro-2-aminothiophenol molecules. In order to improve the yield, a disulfied bond between two 5-nitro-2-aminothiophenol molecules was used as the starting material for the synthesis yielding product (27). The ring of 6-Nitrobenzothiazole (24) was opened by the addition of hydrazine monohydrate in ethanol and heating at
100 °C for 18h then the reaction was exposed to oxygen in the fume hood to oxidize the sulfur which generated a disulfide bond between two 5-nitro-2-aminothiophenol molecules, \(1-(5\text{-nitro-2-aminothiophenol})-5\text{-nitro-2-aminothiophenol}\) \(25\). Reducing the disulfide bond was accomplished immediately via either DTT, \(\text{PPh}_3\), or \(\text{Et}_3\text{P}\). Using triphenylphosphine produced triphenylphosphine oxide which is not easy to isolate from the target product, whereas using DTT or \(\text{PEt}_3\) we were able to reduce the disulfide bond easily and this gave access to the intermediate 5-nitro-2-aminothiophenol \(26\). By adding 5-Bromouracil and \(\text{Na}_2\text{CO}_3\) into the previous mixture and heating at 120 °C for 17 h coupling was achieved yielding product \(27\). The synthesis of the base \(\text{tC}_{\text{nitro}}\) \(28\) was the most challenging that we faced in the course of this lab work. The synthesis using the same pathway that has been used in an article that was published by Börjesson\(^4\) did not show any reaction even after heating the mixture at 116 °C for two days. Also, there was no progress of the reaction after heating the mixture at 127 °C for ~48h. Fortunately, the synthesis of the base \(\text{tC}_{\text{nitro}}\) \(28\) was achieved by using the same pathway that has been used previously for synthesizing the \(\text{tC}_{8\text{OMe}}\) base but by heating product \(27\) in butanol at a higher temperature (170 °C) for 3 days producing a crude mixture containing
the target product (28) as was confirmed via mass spectral analysis. We did not try to purify the product yet, but someone from the group will do so in order to glycosylate the pure base and continue this analog synthesis.
Reduction agent: DTT, PEt₃, or PPh₃
Solvent: n-butanol or ethanol

Scheme 3.2.4: The synthetic pathway to tGₙNucleobase
3.6 Photophysical properties of the fluorescent nucleosides

To understand the effects of the donor substituent group and their positions on the photophysical properties of the tC derivatives, and also to explore whether the features of the fluorescent nucleosides tC derivatives make them ideal for utilization as probes, and for further use in FRET experiments, the photophysical properties of the fluorescent nucleosides tC derivatives have been investigated.

3.6.1 The Absorption of tC, tC\textsubscript{8OMe}, tC\textsubscript{7OMe}:

Figure 3.6.1 shows the normalized absorption spectra of the tC, tC\textsubscript{8OMe}, and tC\textsubscript{7OMe} nucleoside analogues as a dilute solution in Ethanol (\sim 10\mu M). The tC\textsubscript{8OMe} analogue, shows a red shift absorption that peaks at approximately 377 nm whereas the tC\textsubscript{7OMe} spectrum appears as two peaks one at approximately 325 nm and an unclear peak at approximately 374 nm, and the tC shows absorption that peaks at approximately 371 nm. On the other hand, the spectra of the absorption measurements of the tC nucleoside derivatives as dilute (\sim 10 \mu M) solutions in water shows that the tC\textsubscript{8OMe} is the most red shifted absorption that peaks at 381 nm while the tC\textsubscript{7OMe} spectrum shows two peaks at approximately 327 nm and 376 nm,
and the tC shows absorption maximum at 377 nm. Consequently, the absorption of each nucleoside, tC, tC_{8OMe}, and tC_{7OMe}, is minimally sensitive to changes in solvent polarity as is clear in table 3.6.1.

![Normalized Absorption Spectra of tC Derivatives in H_2O](image)

**Figure 3.6.1:** The normalized absorption spectra of tC, tC_{8OMe}, and tC_{7OMe} in EtOH and in H_2O

### 3.6.2 The Fluorescence Emission and Excitation of tC derivatives:

Figure 3.6.2a shows the normalized excitation and emission spectra of the tC, tC_{8OMe}, and tC_{7OMe} nucleosides in ethanol. Upon excitation at 381 nm of a dilute solution (10 µM) in ethanol, the nucleoside tC_{8OMe} displays an emission that peaks at ~518 nm. On the other hand, the nucleosides tC_{7OMe} and tC exhibit shorter emission wavelengths that peak at ~498 nm, and ~483 nm respectively.
The emission measurements were made with different concentrations (between 1µM to 5 µM) in EtOH for each nucleoside and the emission wavelengths were: tC 485.07-488.05 nm, tC$_{8OMe}$ 512.98 - 509.85 nm, and tC$_{7OMe}$ 494.09-499.09 nm.

Figure 3.6.2b shows the normalized excitation and emission spectra of the tC, tC$_{8OMe}$, and tC$_{7OMe}$ nucleosides in ethanol. A dilute solution (10 µM) in water, the nucleoside tC$_{8OMe}$ displays an emission that peaks at ~536 nm. On the other hand, the nucleosides tC$_{7OMe}$ and tC exhibit shorter emission wavelengths that peak at ~517 nm, and ~501 nm respectively.

![Excitation and Emission spectra of tC derivatives in Ethanol](image)

**Figure 3.6.2a:** The excitation and emission spectra of tC, tC$_{8OMe}$, tC$_{7OMe}$ nucleosides in Ethanol (conc. 10µM)
Figure 3.6.2b: The excitation and emission spectra of tC, tC$_{8OMe}$, tC$_{7OMe}$ nucleosides in H$_2$O (conc. 10µM)

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Solvent</th>
<th>$\lambda$$_{em}$ nm</th>
<th>$\lambda$$_{abs}$ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>tC</td>
<td>H$_2$O</td>
<td>501.9</td>
<td>377</td>
</tr>
<tr>
<td>tC</td>
<td>EtOH</td>
<td>482.9</td>
<td>371</td>
</tr>
<tr>
<td>tC$_{7OMe}$</td>
<td>H$_2$O</td>
<td>517.0</td>
<td>376 &amp; ~ 327</td>
</tr>
<tr>
<td>tC$_{7OMe}$</td>
<td>EtOH</td>
<td>497.8</td>
<td>374 &amp; 325</td>
</tr>
<tr>
<td>tC$_{8OMe}$</td>
<td>H$_2$O</td>
<td>536.9</td>
<td>381</td>
</tr>
<tr>
<td>tC$_{8OMe}$</td>
<td>EtOH</td>
<td>517.9</td>
<td>377</td>
</tr>
</tbody>
</table>

Table 3.6.1: Absorption and Fluorescence emission maxima of tC derivatives in H$_2$O and in EtOH

3.6.3 Quantum yields

Quantum yields were determined using anthracene ($\Phi_F$=0.27 in ethanol) as a standard$^7$ for comparison to the nucleosides tC, tC$_{8OMe}$, and tC$_{7OMe}$. The quantum yields of all nucleosides (tC, tC$_{8OMe}$, and tC$_{7OMe}$) were determined using the following equation:
\[ \phi_X = \phi_S \left[ \frac{\text{Grad}_X}{\text{Grad}_S} \right] \frac{\square_x}{\square_S} \]

Equation 1

Where \( \Phi_X \) is the unknown quantum yield of tC the nucleoside analogue. \( \Phi_S \) is the quantum yield of anthracene. Grad is the slope from the plot of the integrated fluorescence intensity versus the absorbance at the excitation wavelength. \( \square_x = \square_S \) is the refractive index of the solvent (1.36 for ethanol).

The nucleosides tC\textsubscript{8OMe}, tC\textsubscript{7OMe} and tC exhibits fluorescence quantum yields in ethanol of \( \phi_F = 0.43, 0.86, 0.17 \) respectively.

**Figure 3.6.3a:** Plot of integrated emission versus absorption to determine the quantum yield of tC
3.6.4 Molar Extinction Coefficients:

The molar extinction coefficients of all nucleosides (tC, tC$_{8OMe}$, and tC$_{7OMe}$) were measured using a Varian Cary 100 Bio Spectrophotometer, and determined using the Beer’s Law equation:
\[ A_\lambda = \varepsilon c L = \varepsilon c \text{ when } L = 1 \text{ cm} \]  

Equation 2

Where, \( \varepsilon \): molar absorptivity, \( A \): absorbance, and \( c \): molar concentration.

The molar absorptivities for tC, tC\(_{80Me}\), and tC\(_{70Me}\) were calculated via the slope of a plot of absorption versus concentration (Figure 3.7.4) to be 8300 M\(^{-1}\) cm\(^{-1}\), 5200 M\(^{-1}\) cm\(^{-1}\) and 5400 M\(^{-1}\) cm\(^{-1}\) respectively.

**Figure 3.6.4:** Plot of absorption versus concentration for tC analogues

From the molar absorptivity we can calculate another quantification of the fluorescence which is brightness.
Brightness = $\varepsilon \times \phi_F \quad \text{Equation 3}$

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Solvent</th>
<th>Quantum Yield in EtOH</th>
<th>Molar absorptive $(M)^{-1} cm^{-1}$</th>
<th>Brightness $(M)^{-1} cm^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>tC</td>
<td>EtOH</td>
<td>0.17</td>
<td>8300</td>
<td>1411</td>
</tr>
<tr>
<td>tC$_{8OMe}$</td>
<td>EtOH</td>
<td>0.43</td>
<td>5200</td>
<td>2080</td>
</tr>
<tr>
<td>tC$_{7OMe}$</td>
<td>EtOH</td>
<td>0.86</td>
<td>5400</td>
<td>4644</td>
</tr>
</tbody>
</table>

Table 3.6.2: Fluorescence quantum yields, molar absorptivity, and brightness in EtOH

3.7 Experimental

Synthesis of tC nucleoside analogue derivatives and the nucleobase tC$_{\text{nitro}}$

General

Solvents and reagents were obtained from commercial suppliers and used without further purification. All moisture and oxygen sensitive reactions were carried out under nitrogen. Air and moisture sensitive solvents and solutions of reactions were transferred by syringe. Reactions were monitored by thin layer chromatography (TLC). NMR spectra were recorded on a Bruker Avance 500MHz spectrometer. UV-Vis absorption spectra were recorded on a Varian Cary 100 Bio Spectrophotometer, and fluorescence, excitation, and emission spectra were recorded on a Varian Cary Eclipse Fluorimeter. Mass spectra were measured at the Central Analytical Laboratory at the University of Colorado at Boulder. Unless otherwise
specified, purifications were performed using Teledyne Isco advanced automated chromatography CombiFlash® Rf with ultra pure flash column silica gel.

![Scheme 3.7.1: The synthesis of 2,4-Dihydroxy-5-(2-amino-phenylthio)pyrimidine](image)

**Scheme 3.7.1:** The synthesis of 2,4-Dihydroxy-5-(2-amino-phenylthio)pyrimidine

2,4-Dihydroxy-5-(2-amino-phenylthio)pyrimidine(3).

2-aminothiophenol (1) (22.33 mmol, 2.8 g) was dissolved in ethylene glycol (10 mL) under nitrogen gas. 5-bromouracil (2) (16.6 mmol, 3.16 g) and Na₂CO₃ (16.6 mmol, 1.76g) were added to the solution. The mixture was heated at 120°C for an hour then was allowed to cool to room temperature before adding water (30 mL) then the reaction was neutralized by dropwise addition of ~2 mL acetic acid pH indicator paper was used to check that the neutralization was complete and the pH of the mixture was 7. A yellow precipitate was collected via filtration and washed first with water and then ethanol until the precipitate had become colorless. The precipitate was finally washed with a small volume of ether to facilitate drying. The precipitate was dissolved in warm 0.02 M Sodium hydroxide (33 mL)
warmed to ~50°C then the mixture was neutralized to pH ~7 with acetic acid. The mixture was filtered and the white precipitate was washed first with water, then with ethanol and finally with ether. A white precipitate formed which was product (3) (2.2 g) in 62% yield. $^1$H NMR (500 MHz, DMSO-d$_6$, 294 K): $\delta$ 11.36 (1 H, s), 11.16 (1 H, s), 7.53 (1 H, d, $J$ 1.6), 7.31 (1 H, dt, $J$ 7.7, 1.7), 7.08 – 7.03 (1 H, m), 6.70 (1 H, dt, $J$ 8.2, 1.5), 6.50 (1 H, tt, $J$ 7.4, 1.6), 5.52 (2 H, s).

![Chemical structure of 1,3-diaza-2-oxophenothiazine (tC base) (4).](image)

Scheme 3.7.2: The synthesis of tC base

1,3-diaza-2-oxophenothiazine (tC base) (4).

2,4-Dihydroxy-5-(2-amino-phenylthio)pyrimidine (3) (3.625 mmol, 0.853 g) was stirred in ethanol (17.3 mL) then HCl (1.73 mL) was added dropwise. The reaction mixture was stirred at 80 °C for 22 h. The reaction mixture was cooled to room temperature and filtered. The precipitate was added to a stirred, warm (50 °C) aqueous 5% ammonia solution. After 5 min the solution was filtered. This washing procedure was repeated twice. The yellow product was finally washed with water and left to dry overnight to
yield compound (4) in 0.575 g, 73% yield. $^1$H-NMR (500 MHz, DMSO-d6, 294 K): $\delta$ 10.24 (1 H, s), 7.42 (1 H, s), 7.10 – 7.00 (2 H, m), 6.92 (2 H, d, $J$ 8.0).

Scheme 3.7.3: The synthesis of 2´-deoxy-3´,5´-di-O-p-toluoyl-phenothiazine

2´-deoxy-3´,5´-di-O-p-toluoyl-phenothiazine (5).

1,3-diaza-2-oxophenothiazine (tC base) (4) (0.299 mmol, 0.065 g) was stirred in anhydrous acetonitrile (1.4 mL, 99%) for 10 min at room temperature under nitrogen. Bis(trimethylsilyl)acetamide (0.102 mL) was added to the solution. After 40 min of stirring at room temperature 3´,5´-bis(toluoyl)-2-deoxyribosyl chloride (0.383 mmol, 0.149 g) was added at $0^\circ$C followed by (6 µ1) SnCl$_4$ dropwise. After 4 h, 2´-deoxy-3´,5´-di-O-p-toluoyl-phenothiazine (5) was generated. The progress of the reaction was monitored by TLC. The mixture was diluted with ethyl acetate (8 mL) and the resulting solution was washed with saturated NaHCO$_3$ (3x20 mL) and
brine (1x20 mL). The organic layer was dried over anhydrous Na₂SO₄, and concentrated using a rotary evaporator. Purification via flash column chromatography on silica gel (CH₂Cl₂: MeOH, 100:0 → 0:100) afforded the desired product (5) in a mixture of α and β anomers, 0.068g, 40 % yield.

**¹H NMR (500 MHz, Chloroform-d, 294 K):** δ 7.92 – 7.88 (3 H, m), 7.76 – 7.70 (1 H, m), 7.43 (1 H, d, J 12.4), 7.07 (1 H, dd, J 8.0, 7.1, 1.8), 7.00 – 6.97 (3 H, m), 6.93 – 6.89 (1 H, m), 6.86 (1 H, dd, J 8.0, 1.2), 6.36 (1 H, dd, J 8.2, 5.6), 6.30 (1 H, dd, J 6.7, 1.1), 5.64 – 5.57 (2 H, m), 5.53 (1 H, dd, J 8.1, 6.2, 1.9), 4.88 (1 H, t, J 4.3), 4.78 (1 H, dd, J 12.2, 3.0), 4.66 (1 H, dd, J 12.3, 3.3), 4.59 (1 H, q, J 2.9), 4.55 – 4.51 (2 H, m), 4.45 – 4.40 (1 H, m), 3.49 (1 H, s), 2.99 – 2.88 (2 H, m), 2.62 (1 H, d, J 15.4), 1.98 (2 H, d, J 18.7).

[Scheme 3.7.4: The synthesis of 3-(2-Deoxy-β-D-ribofuranosyl)-1,3-diaza-2-oxopenthiazine]
3-(2-Deoxy-b-D-ribofuranosyl)-1,3-diaza-2-oxophenothiazine (6) & (7).

2´-deoxy-3´,5´-di-O-p-toluoyl-phenothiazine (5) (0.478 mmol, 0.159 g) was dissolved in (5 mL) absolute methanol at room temperature. NaOMe (30% wt. 5.4 M, 99% in methanol) (0.1 mL) was added dropwise to the solution. The progress of the reaction was monitored by TLC. After 30 min the reaction was complete, and drops of acetic acid were added to quench the reaction. The solvent was removed by evaporation using a rotary evaporator. Purification via flash column chromatography on silica gel (CH$_2$Cl$_2$: MeOH, 100:0 → 45:55) afforded free isolated nucleoside anomers $\beta$ (6) and $\alpha$ (7) that were identified depending on the elution profile from automated chromatography using an Isco gradient (CombiFlash® Rf ) and confirmed via $^1$H NMR 500. The combined yield is 0.0685 g, 43 %.

$\beta$-anomer

$^1$H NMR (500 MHz, Methanol-d4, 294 K): $\delta$ H (500 MHz, Methanol-d4)

7.93 (1 H, s), 7.91 – 7.86 (1 H, m), 7.26 (1 H, d, J 8.0), 7.07 (1 H, td, J 7.7, 1.5), 6.99 (1 H, dd, J 7.9, 1.5), 6.96 – 6.92 (1 H, m), 6.85 (1 H, dd, J 8.1, 1.1), 6.18 (1 H, t, J 6.3), 4.36 (1 H, dt, J 6.4, 4.0), 3.93 (1 H, q, J 3.5), 3.81 (1 H, d, J 3.1), 3.74 (1 H, d, J 3.6), 2.40 (2 H, s), 2.14 (1 H, dt, J 13.3, 6.5).
\( \alpha \)-anomer

\(^1\)H (500 MHz, Methanol-d4, 294 K) \( \delta \) 7.69 (1 H, s), 7.10 – 7.03 (1 H, m), 7.00 (1 H, dd, J 7.8, 1.5), 6.94 (1 H, td, J 7.5, 1.3), 6.86 (1 H, d, J 1.3), 6.84 (1 H, d, J 1.3), 6.09 (1 H, dd, J 7.2, 2.1), 4.35 – 4.30 (3 H, m), 3.63 – 3.55 (3 H, m), 2.70 – 2.62 (1 H, m), 2.08 (1 H, dt, J 14.6, 2.1).

\[ \text{O} \quad \text{N} \quad \text{S} \quad \text{H} \quad \text{O} \]

\[ \text{O} \quad \text{S} \quad \text{S} \quad \text{NH}_2 \quad \text{NH}_2 \]

\[ \text{H}_3\text{CO} \quad \text{SH} \quad \text{NH}_2 \]

Scheme 3.7.5: The synthesis of 4-methoxy-2-aminothiophenol

1-(4-methoxy-2-aminothiophenol)-4-methoxy-2-amino-thiophenol(9).

5-methoxy-2-methyl benzothiazole (8) (5.57mmol, 1g) was dissolved in ethylene glycol (6 mL) under nitrogen gas. NaOH (6 mL, 10 M) was added and the mixture was heated at 125°C overnight. Unfortunately, the product mixture contained more 1-(4-methoxy-2-aminothiophenol)-4-methoxy-2-aminothiophenol without nitrogen gas followed by the addition of \( \text{H}_2\text{O}_2 \) after cooling of the reaction to room temperature to increase the chance of
oxidizing the sulfur bond. The yellow product 1-(4-methoxy-2-aminothiophenol)-4-methoxy-2-aminothiophenol, disulfide (9) was isolated via flash column chromatography on silica gel (CH$_2$Cl$_2$: MeOH, 100:0 → 95:5) in 0.859 g, 68% yield, which is more than the target product 2-amino-4-methoxythiophenol (10). As a result, we decided to prepare product (9) by the same reaction conditions.

\[ \text{Scheme 3.7.6: The synthesis of 2,4-Dihydroxy-5-(2-amino-4-methoxyphenylthio)pyrimidine} \]
2,4-Dihydroxy-5-(2-amino-4-methoxy-phenylthio)pyrimidine (11).

1-(4-methoxy-2-aminothiophenol)-4-methoxy-2-aminothiophenol (9) (1.02 mmol, 0.316 g) was dissolved in diglyme (4 mL), and was reduced to 2-amino-4-methoxythiophenol (10) by heating with dithiothreitol, DTT, (1.02 mmol, 0.158 g) under nitrogen at 80 °C. After 30 min 5-Bromouracil (2.1 mmol, 0.416 g) and Na₂CO₃ (2.04 mmol, 0.216 g) were added and the mixture was heated at 120 °C 2 h. The solvent was removed by evaporation using rotary evaporator. Purification via flash column chromatography on silica gel (5% methanol in dichloromethane) afforded the desired product (11) in 0.18 g, 33% yield. 

\(^1\)H NMR (500 MHz, DMSO-d₆, 294 K): δ 11.33 (2 H, s), 7.27 (1 H, d, J 8.5), 6.27 (1 H, d, J 2.7), 6.12 (1 H, d, J 2.7), 6.11 (1 H, d, J 2.7), 5.60 (2 H, s), 3.66 (3 H, s).

Scheme 3.7.7: The synthesis of 2′-deoxy TC₆GOME base

85
8-methoxy-1,3-diaza-2-oxophenothiazine nucleoside (tC$_{8OMe}$ base) (12).

2,4-Dihydroxy-5-(2-amino-4-methoxy-phenylthio)pyrimidine (11) (0.49 mmol, 0.13 g) was dissolved in butanol (1 mL). While the mixture was stirring, HCl (0.5 mL, 37%) was added dropwise. The reaction mixture was heated at 116°C. After 22 h the mixture was cooled to room temperature and the solvent was removed by evaporation via rotary evaporation. Purification via flash column chromatography on silica gel (8% methanol in dichloromethane) afforded the desired product (12) in 0.036 g, 30% yield.

$^1$H-NMR (500 MHz, DMSO-d$_6$, 294 K): $\delta$ 11.04 (1 H, s), 10.16 (1 H, s), 7.42 (1 H, s), 6.96 (1 H, d, J 8.5), 6.58 (1 H, s), 6.53 (1 H, dd, J 8.6, 2.7), 3.68 (3H, s). (ESI-TOF) m/z: calcd. for C$_{11}$H$_9$N$_3$O$_3$S [M+H] 248.29 found 248.0. ESI-TOF) m/z: calcd. for C$_{11}$H$_9$N$_3$O$_3$S [M+Na] 270.26 found 270.0.

Scheme 3.7.8: The synthesis of 2'-deoxy-3',5'-bis-O-p-toluoyl-8-methoxy-phenothiazine
2′-deoxy-3′,5′-bis-O-p-toluoyl-8-methoxy-phenothiazine (13).

8-methoxy-1,3-diaza-2-oxophenothiazine nucleoside (tC8OMe base) (12) (0.877 mmol, 0.2 g) was stirred in anhydrous acetonitrile 99% (2.12 mL) at room temperature under nitrogen for 10 min. Bis(trimethylsilyl)acetamide (0.3 mL) was added to the solution. After 40 min, the reaction was cooled to 0 °C. 3′,5′-bis(toluoyl)-2-deoxyribosyl chloride (1.13 mmol, 0.44 g) was added, and followed by dropwise addition of SnCl4 (6 µl). The progress of the reaction was monitored by TLC. The reaction was completed after 4 h. The mixture was dissolved in ethyl acetate (3 mL), and the resulting solution was washed with saturated NaHCO3 (3x20 mL) and brine (1x20 mL). The organic layer was dried over anhydrous Na2SO4, and concentrated using a rotary evaporator. Purification via flash column chromatography on silica gel (100:0 →60:40, CH2Cl2: MeOH) afforded the desired product (13) as a mixture of β and α anomers ~ 1:1, 0.21 g, 40% yield.

1H NMR (500 MHz, Chloroform-d, 294 K): δ 7.94 (2 H, d, J 1.5), 7.93 (2 H, d, J 1.4), 7.90 (1 H, d, J 1.6), 7.88 (1 H, d, J 1.7), 7.73 (1 H, d, J 1.8), 7.72 (1 H, d, J 1.8), 7.46 (1 H, s), 7.44 (1 H, s), 7.21 (1 H, s), 7.20 (1 H, s), 7.02 (1 H, s), 7.00 (1 H, s), 6.88 (1 H, d, J 8.6), 6.81 (1 H, d, J 8.5), 6.71 (1 H, d, J 2.6), 6.59 (2 H, t, J 2.7), 6.57 (1 H, d, J 2.5), 6.55 (1 H, dd, J 8.5,
2.5), 6.33 (1 H, dd, J 8.0, 5.5), 6.28 (1 H, d, J 6.5), 5.62 (1 H, d, J 5.8), 5.59 (1 H, d, J 6.4), 4.89 (1 H, t, J 4.3), 4.78 (1 H, dd, J 12.3, 2.9), 4.66 (1 H, dd, J 12.2, 3.3), 4.60 (1 H, q, J 2.8), 4.54 – 4.51 (2 H, m), 3.79 (3 H, s).

Scheme 3.7.9: The synthesis of 3-(2-Deoxy-β-D-ribofuranosyl)-8-methoxy-1,3-diaza oxo-phenothiazine

3-(2-Deoxy-β-D-ribofuranosyl)-8-methoxy-1,3-diaza-oxo-phenothiazine (14) & (15).

2´-deoxy-3´,5´-bis-O-p-toluoyl-8-methoxy-phenothiazine (13) (0.35 mmol, 0.209 g) was dissolved in absolute methanol (5 mL) at room temperature. NaOMe (30% wt. 5.4 M, 99% in methanol) (0.1 mL) was added dropwise to the solution. After 30 min the reaction was complete, and drops of acetic acid were added to quench. The solvent was removed by evaporation using a rotary evaporator. Purification via flash column chromatography on silica gel (CH₂Cl₂: MeOH, 100:0 → 55:45) afforded free
isolated nucleoside anomers (14) & (15) β and α respectively that were identified depending on the elution profile from automated chromatography using an Isco gradient (CombiFlash® Rf ) and confirmed via $^1$H NMR 500. The combined yield is 0.042 g, 34 %.

β anomer

$^1$H NMR (500 MHz, Methanol-d$_4$, 294 K): δ 7.93 (1 H, s), 6.91 (1 H, d, J 8.6), 6.58 (1 H, d, J 2.6), 6.56 (1 H, d, J 2.6), 6.51 (1 H, d, J 2.5), 6.19 (1 H, t, J 6.4), 4.93 (1 H, s), 4.85 (1 H, s), 4.61 (1 H, s), 4.37 – 4.35 (1 H, m), 3.93 (1 H, p, J 3.5), 3.82 (1 H, dd, J 12.1, 3.1), 3.75 (3 H, s), 2.36 (2 H, ddd, J 13.6, 6.1, 4.0), 2.17 – 2.15 (1 H, m), 2.15 – 2.11 (1 H, m).

α anomer

$^1$H NMR (500 MHz, Methanol-d$_4$, 294 K): δ 7.69 (1 H, s), 6.93 – 6.89 (1 H, m), 6.57 (1 H, dd, J 8.6, 2.6), 6.51 (1 H, d, J 2.6), 6.09 (1 H, dd, J 7.2, 2.1), 4.81 (1 H, s), 4.61 (3 H, s), 4.34 (2 H, dd, J 4.7, 3.4), 3.80 (1 H, s), 3.75 (3 H, s), 2.71 – 2.62 (1 H, m), 2.09 (1 H, s), 2.06 (1 H, s).
Scheme 3.7.10: The synthesis of 1-(5-methoxy-2-aminothiophenol)-5-methoxy-2-amino thiophenol

1-(5-methoxy-2-aminothiophenol)-5-methoxy-2-amino-thiophenol (17).

Hydrazine monohydrate (10 mL) was added to 6-methoxy-2-methyl-benzothiazole (16) (5.58 mmol, 1g). The mixture was heated at 100°C for 22 h. Hydrogen peroxide was added dropwise and the mixture was left to evaporate in the hood two days. The crude product was dissolved in methanol and precipitated by the addition of water. The afforded yellow precipitate was collected via filtration and washed with water affording the desired product (17) in 0.504 g, 60% yield. $^1$H NMR (500 MHz, DMSO-d6, 294 K): δ 6.77 (1 H, dd, J 8.7, 2.9), 6.70 (1 H, d, J 8.8), 6.59 (1 H, d, J 2.9), 5.04 (2 H, s), 3.52 (3 H, s).
2,4-Dihydroxy-5-(2-amino-5-methoxy-phenylthio)pyrimidine (19).

1-((5-methoxy-2-aminothiophenol)-5-methoxy-2-aminothiophenol (17) (1.67 mmol, 0.515g) was dissolved in diglyme (10 mL). Triethylphosphine in 1 M tetrahydrofuran (1.669 mL) and water (0.03 mL) were added to reduce the disulfide bond and afforded 2-amino-5-methoxythiophenol (18) at room temperature. After 30 min, 5-Bromouracil (3.34 mmol, 0.637g) and Na$_2$CO$_3$ (3.34 mmol, 0.353 g) were added. The mixture was heated at 120°C for 1h. The solvent was removed by evaporation using a rotary evaporator. Purification via flash column chromatography on silica gel (CH$_2$Cl$_2$: MeOH, 100:0 → 20:80) afforded the desired product (19) in 0.307 g, 35% yield.$^1$H-NMR (500 MHz, DMSO-d$_6$, 294 K): δ 11.28 (2 H, d, J 74.4), 7.59 (1 H, s), 6.89 (1 H, d, J 2.9), 6.72 (1 H, dd, J 8.8, 2.9), 6.65 (1 H, d, J 8.7), 5.05 (2 H, s), 3.62(3 H,s)
7-methoxy-1,3-diaza-2-oxophenothiazine nucleoside (tC\textsubscript{7OMe} base) (20).

2,4-Dihydroxy-5-(2-amino-5-methoxy-phenylthio)pyrimidine (19) (0.58 mmol, 0.154 g) was stirred in butanol (10 mL) at 100°C for 10 min. The mixture was cooled to room temperature, followed by dropwise addition of HCl (1 mL, 37%). The reaction mixture was heated overnight at 116°C, an orange precipitate was generated which became yellow after washing with water yielding 0.087g, 61%. tC\textsubscript{7OMe} base (20). No further purification was necessary for the tC\textsubscript{7OMe} base (20). \textsuperscript{1}H-NMR (500 MHz, DMSO-d\textsubscript{6}, 294 K): δ 10.90 (1 H, s), 10.11 (1 H, s), 7.38 (1 H, s), 6.84 (1 H, d, J 8.6), 6.65 (2 H, d, J 8.7), 3.67 (3 H, s). (ESI-TOF) m/z: calcd. for C\textsubscript{11}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2}S [+H\textsuperscript{+}] 248.29
found 248.1. (ESI-TOF) m/z: calcd. for C\textsubscript{11}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2}S \[+Li^+\] 254.21 found 254.1. (ESI-TOF) m/z: calcd. for C\textsubscript{11}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2}S \[Na^+\] 270.26 found 270.0.

Scheme 3.7.13: The synthesis of 2´-deoxy-3´,5´-bis-O-p-toluoyl-7-methoxy-phenothiazine (21).

\( \text{2´-deoxy-3´,5´-bis-O-p-toluoyl-7-methoxy-phenothiazine (21).} \)

7-methoxy-1,3-diaza-2-oxophenothiazine nucleoside (tC\textsubscript{7OMe} base) (20) (0.39 mmol, 0.096 g) was dissolved in anhydrous acetonitrile 99% (1 mL) at room temperature under nitrogen for 10 min. Bis(trimethylsilyl)acetamide (0.133 mL) was added to the solution. After 40 min stirring at room temperature, 3´,5´-bis(toluoyl)-2-deoxyribosyl chloride (0.504 mmol, 0.194 g) was added at 0º C followed by dropwise addition of SnCl\textsubscript{4} (8.34 µl). The progress of the reaction was monitored by TLC. The reaction was completed after 4 h, the mixture was dissolved in ethyl acetate, and the resulting solution was washed with saturated NaHCO\textsubscript{3}
(3x20 mL) and brine (1x20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, and concentrated by evaporation of the solvent by a rotary evaporator. Purification via flash column chromatography on silica gel (100:0 → 60:40, CH$_2$Cl$_2$ : MeOH) afforded the desired product (21) in mixture of α and β anomers 0.1093 g, 47% yield. $^1$H-NMR (500 MHz, Chloroform-d, 294 K): δ 8.03 (2 H, d, J 7.9), 7.96 – 7.92 (4 H, m), 7.91 – 7.88 (2 H, m), 7.75 – 7.73 (1 H, m), 7.21 (2 H, d, J 8.1), 7.02 (1 H, d, J 7.9), 6.87 (0 H, d, J 8.7), 6.79 (1 H, d, J 8.7), 6.67 (1 H, dd, J 8.7, 2.7), 6.61 (1 H, dd, J 8.7, 2.7), 6.53 (0 H, d, J 2.7), 6.46 (1 H, d, J 2.7), 6.35 (1 H, dd, J 8.2, 5.5), 6.29 (1 H, d, J 6.4), 5.63 – 5.56 (2 H, m), 4.88 (1 H, t, J 4.2), 4.78 (1 H, dd, J 12.3, 3.0), 4.66 (1 H, dd, J 12.2, 3.3), 4.58 (1 H, q, J 2.8), 4.55 – 4.51 (1 H, m), 3.75 (3 H, s), 3.35 (1 H, s).

![Scheme 3.6.14: The synthesis of 3-(2-Deoxy-b-D-ribofuranosyl)-7-methoxy-1,3-diaza oxo-phenotheazin](image)
3-(2-Deoxy-b-D-ribofuranosyl)-7-methoxy-1,3-diaza-2-oxo-phenothiazine (22) & (23)

NaOMe (30% wt. 5.4 M, 99% in methanol) (0.2 mL) was added dropwise to 2´-deoxy-3´,5´-bis-O-p-toluoyl-7-methoxy-phenothiazine (21) (0.0233 mmol, 0.014 g) dissolved in methanol (3 mL) at room temperature. After 30 min the reaction was complete, and drops of acetic acid were added to quench the reaction then the solvent was removed by evaporation with a rotary evaporator. Purification via flash column chromatography on silica gel (CH$_2$Cl$_2$: MeOH, 100:0 → 60:40) afforded isolated free nucleoside anomers β and α that were identified depending on the elution profile from automated chromatography using an Isco gradient (CombiFlash® Rf) in yield (~ 9.46 % β & 4.72 % α and mixture α >> β yield 43.7%) and confirmed via $^1$H NMR 500. In combined is yield 0.0057 g, 67 %. (ESI-TOF) m/z: calcd. for C$_{32}$H$_{29}$N$_3$O$_7$S [+H$^+$] 600.66 found 600.2. (ESI-TOF) m/z: calcd. for C$_{32}$H$_{29}$N$_3$O$_7$S [+Li$^+$] 606.59 found 606.2. (ESI-TOF) m/z: calcd. for C$_{32}$H$_{29}$N$_3$O$_7$S [+Na$^+$] 622.64 found 622.2.
2,4-Dihydroxy-5-(2-amino-5-nitro-phenylthio)pyrimidine (tC\text{nitro}) (27).

6-Nitrobenzothiazole (24) (13.87 mmol, 2.5 g) was dissolved in ethanol (28 mL). After 5 min, hydrazine monohydrate (28 mL) was added to the solution. The reaction mixture was heated at 100 °C for 18h. The 1-(5-nitro-2-aminothiophenol)-5-nitro-2-aminothiophenol, nitro-disulfide, (25) was generated and collected via filtration then washed with water to afford the yellow product in around ~100% yield. \( ^1H \text{NMR} \) (500 MHz, DMSO-d6, 294 K): δ 7.99 (1 H, dd, J 9.2, 2.7), 7.49 (1 H, d, J 2.7), 7.23 (2 H, s), 6.85 (1 H, d, J 9.2). 1-(5-nitro-2-aminothiophenol)-5-nitro-2-aminothiophenol, nitro-disulfide, (25) (0.65 mmol, 0.22 g) was dissolved in diglyme (8 mL). Dithiothreitol (1.08 mmol, 0.167 g) and water (0.03 mL) were added to reduce the disulfide bond, and the reaction mixture was heated at 80°C to afford 5-nitro-2-aminothiophenol (26). 5-Bromouracil (1.3 mmol, 0.248 g)
and Na$_2$CO$_3$ (1.3 mmol, 0.137 g) were added. The mixture was heated at 120°C for 17 h. The mixture was left to evaporate in the hood two days. Purification via flash column chromatography on silica gel (5% methanol in dichloromethane) afforded the desired product (27) in 0.156 g, 71 % yield.

$^1$H-NMR (500 MHz, DMSO-d$_6$, 294 K): $\delta$ 8.24 (2 H, d, J 2.7), 8.06 (2 H, s), 6.74 (1 H, s), 6.73 (1 H, s), 5.76 (2 H, s). (ESI-TOF) m/z: calcd. for C$_{12}$H$_{10}$N$_4$O$_4$S$_2$ [+H$^+$] 281.27 found 281.0. (ESI-TOF) m/z: calcd. for C$_{12}$H$_{10}$N$_4$O$_4$S$_2$ [+K$^+$] 319.36 found 319.0. (ESI-TOF) m/z: calcd. for C$_{12}$H$_{10}$N$_4$O$_4$S$_2$ [+Na$^+$] 303.25 found 303.0.

![Scheme 3.7.16: The synthesis of $t$C$_{nitro}$ base](image)

7-nitro-1,3-diaza-2-oxophenothiazine nucleobase ($t$C$_{nitro}$ base) (28).

2,4-Dihydroxy-5-(2-amino-5-methoxy-phenylthio)pyrimidine (27) (0.082 mmol, 0.23 g) was stirred in butanol (1.15 mL) followed by dropwise
addition of HCl (0.4 mL, 37%). The reaction mixture was heated 3 days at 170°C. The crude product was left to dry in the hood. The NMR measurement shows the target product (28) was generated. \(^1\text{H-NMR (500 MHz, DMSO-d6, 294 K)}: \delta 7.40 (1 \text{ H, s}), 7.39 (2 \text{ H, d, J 1.8}), 7.37 (1 \text{ H, s}), 5.45 (1 \text{ H, d, J 1.6}), 5.44 (1 \text{ H, d, J 1.8}). (\text{ESI-TOF}) \text{ m/z: calcd. for } C_{10}H_{6}N_{4}O_{3}S [+H] 263.25 \text{ found 263.0. (ESI-TOF) m/z: calcd. for } C_{10}H_{6}N_{4}O_{3}S [M^+] 262.24 \text{ found 262.0. (ESI-TOF) m/z: calcd. for } C_{10}H_{6}N_{4}O_{3}S [+Li^+] 269.19 \text{ found 269.0. (ESI-TOF) m/z: calcd. for } C_{10}H_{6}N_{4}O_{3}S \text{ Na} [+Na^+] 285.23 \text{ found 285.0.}

3.8 Conclusion

We have successfully synthesized two new tC fluorescent nucleosides with methoxy substitutions, tC\textsubscript{8OMe} (Figure 3.2.2), tC\textsubscript{7OMe} (Figure 3.2.3), in addition to preparing tC (Figure 3.2.1) which has been reported previously by Matteucci and co-workers \(^2\), and introduced a new synthetic route to tC\textsubscript{nitro} that previously had been prepared in low yield by Börjesson et. al. 2009.\(^4\)

The fluorescence study on the tC derivatives suggested that the emission spectra in ethanol of tC\textsubscript{8OMe}, with a maximum at ~ 518 nm, has the
greatest red shift, approximately 35 nm as compared to tC with a maximum at ~ 483 nm. The emission spectra of tC7OMe, with a maximum at ~ 498 nm is slightly red-shifted, approximately 15 nm as compared to tC. Likewise, the emission spectrum in water of tC8OMe, with a maximum at ~ 537 nm, has the greatest red shift, approximately 35 nm as compared to tC with a maximum at ~ 502 nm, while the emission spectrum of tC7OMe, with a maximum at ~ 517 nm was slightly red-shifted, approximately 15 nm as compared to tC.

The absorption spectrum of tC8OMe is also red-shifted compared to the absorption spectrum of tC. The absorbance maximum of tC8OMe is generally around 377 nm in ethanol, and 381 nm in water, which means that it is red-shifted approximately 4 nm compared to the absorbance maximum of tC that is generally around 373 nm in ethanol, and 377 nm in water. Whereas the absorbance maximum of tC7OMe is generally around 374 in ethanol, and 376 nm in water, which means that it is red-shifted in ethanol, but blue shifted in water approximately 1 nm compared to tC.

The quantum yields of both tC8OMe and tC7OMe, that were calculated via a plot of integrated emission versus absorption (Figure 3.7.3), were relatively high (0.38, and 0.8 respectively) compared to the tC quantum yield of 0.21.
These results demonstrated that the tC\textsubscript{7OMe}, which has the methoxy donor group \textit{para} to the amino group, has the highest quantum yield and brightness.

To sum up, our results suggest that the different substitution positions create important distinctions between the nucleosides derivatives of tC. The unsubstituted tC nucleoside was easy to synthesize. In contrast, the synthetic strategy of the tC\textsubscript{7OMe} nucleoside that has the methoxy electron donor substituent group located at carbon 7 and \textit{para} to the amino group is synthetically less challenging and higher yielding than the synthesis of tC\textsubscript{8OMe} which has the methoxy group substituent \textit{meta} to the amine group. Moreover, the donor substituent and the position of the substituent have affected the photophysical properties of the fluorescent nucleoside derivatives of tC. The fluorescence studies demonstrated that fluorescence emission and absorption wavelengths for tC\textsubscript{7OMe} and tC\textsubscript{8OMe} were red shifted compared to the unmodified tC. That means, the methoxy donor group which allows for extended conjugation of unshared pairs of electrons leads to an increase in the freedom of the $\pi$-electrons and this is likely to raise the fluorescence. Also, our fluorescence study established that there are effects of the different positions of the substituent donor group. The
fluorescence emission wavelength of tC8OMe, where methoxy is *meta* to the amino group, is red shifted longer than the fluorescence emission wavelength of tC7OMe, where methoxy is *para* to the amino group.
3.9 References


Appendix A

NMR Spectra