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Design, synthesis and incorporation of fluorescent thiophene-modified nucleoside analogs

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Design, Synthesis and Incorporation of Fluorescent Thiophene-Modified Nucleoside Analogs

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

Mary Streid Noé

Committee in Charge:

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Professor William Gerwick
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Professor Teru Nakagawa

2012
The Dissertation of Mary Streid Noé is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012
DEDICATION

Dedicated to the memory of my father,

Dr. Michael James Noé.
What have I learned but
the proper use for several tools?

The moments
between hard pleasant tasks

To sit silent, drink wine,
and think my own kind
of dry crusty thoughts.

– the first Calochortus flowers
and in all the land,
it's spring.

I point them out:
the yellow petals, the golden hairs,
to Gen.

Seeing in silence:
never the same twice,
but when you get it right,

you pass it on.

*Gary Snyder*
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The central dogma of molecular biology asserts the vastly important roles of nucleic acids in all domains of life. In order to explore and illuminate the intricate biomolecular interactions of nucleic acids with themselves and other biomolecules, isomorphic fluorescent nucleoside analogs have been developed. Fluorescence spectroscopy allows for the monitoring of molecular dynamics down to the subnanomolar concentration in real-time.

In order to continue to evolve as a field, novel isomorphic fluorescent nucleoside analogs must be synthesized and implemented in a variety of ways. Thiophene-modified fluorescent nucleoside analogs have proven to be of significant
utility. However, the low quantum yield of several of these modified fluorescent nucleosides leaves room for further experimentation and improvement. To increase fluorescence signal sensitivity for several biophysical assays, multiple incorporations of a single thiophene-modified nucleoside were explored. These resulted in an enhanced on-signal for duplex melting and annealing as well as mismatch dC detection.

Additionally, in order to form pi-expanded chromophores, four new thiophene-modified fluorescent nucleoside analogs base on pyrrolo-cytidine (PyC) were synthesized and evaluated. Although these new nucleosides (fused and extended PyC analogs) are slightly larger than previous generations of Tor lab fluorescent nucleosides, they possess exceptional photophysical qualities such as unusually high brightness and sensitivity towards solvent polarity.

Finally, a novel fluorescent nucleoside analog FRET system was explored in hopes of monitoring RNA helicase activity in real-time. After synthesizing a novel fluorescent nucleic acid construct, a number of important factors were illustrated with regard to the need for a further red-shifted excitation wavelength and a higher quantum yield in the presence of endogenous proteins and their autofluorescence. Further studies into nucleic acid–protein interactions with a fluorescent nucleoside FRET pair hold great promise.
CHAPTER 1: Introduction

1.1 – General background

Data from various analytical techniques have been integrated to advance a fundamental understanding of the structure and properties of nucleic acids. NMR spectroscopy and X-ray crystallography, for example, have supplied invaluable information, especially with regard to “static” structures. Nucleic acids, like all other biomolecules, are highly dynamic, with hybridization and melting, as well as strand cleavage, base flipping, and ligand binding playing important roles in their biological function. Fluorescence based techniques, due to their sensitivity and short time scale, can facilitate the monitoring of such dynamic events.1-3

Because of the extremely low emission quantum yields of the native nucleosides,4 nucleic acids must to be modified with appropriate chromophores to facilitate fluorescence-based measurements. Although end-labeled oligonucleotides have been extensively used, such approaches rarely provide information at the nucleotide level. In contrast, minimally perturbing fluorescent nucleoside analogs, judiciously replacing selected residues, open a window into otherwise spectroscopically-silent molecular events involving nucleic acids.5 Such “isomorphic” fluorescent nucleobases could ideally replace the native heterocycles without impacting their inherent function, while being in proximity to the site of interest. Additionally, tuning the photophysical characteristics of novel nucleobases to match the spectral features of other established fluorophores can facilitate the
implementation of FRET-based assemblies for the study of biological recognition events, with much higher “resolution” compared to assemblies that rely on end-labeled oligonucleotides.\textsuperscript{6-9} In this chapter, we focus on understanding the interactions of this important biomolecule with ligands via the implementation of new fluorescent isomorphic nucleosides. We open with a concise description of relevant fluorescence-based techniques.

1.2 – Fluorescence Spectroscopy

A detailed overview of photoluminescence is beyond the scope of this chapter and the reader is referred to several excellent texts.\textsuperscript{1-3, 10-12} A brief introduction to rudimentary principles of fluorescence spectroscopy and commonly used techniques follows. Fluorescence typically refers to the emission of a photon from an excited singlet state of a chromophore. Population of this excited state is normally achieved by the absorption of electromagnetic radiation of specific energy. In fluorescence spectroscopy, a sample is irradiated with energy in the UV-visible range of the electromagnetic spectrum. The extent of energy absorption depends upon the molar extinction coefficient (\(\varepsilon\)), which is directly proportional to the absorption cross-section (\(\sigma\)) of the chromophore. After the absorption of a photon, an excited molecule may return to its ground state by a variety of nonradiative and radiative processes. The most common processes of energetic relaxation involve molecular vibrations and rotations as well as intermolecular collisions, especially with solvent molecules. Certain molecules, known as fluorophores, are capable of an alternate process of radiative energy decay in which a photon is emitted.
Several parameters provide insight into the characteristics of a fluorophore and its potential utility. The fluorescence quantum yield ($\Phi_F$) is defined as the fraction of photons emitted versus the number of photons absorbed and ranges from zero to unity. The brightness of a fluorophore is the product of the molar absorptivity ($\varepsilon$) and the fluorescence quantum yield ($\Phi_F$). The fluorescence lifetime of a chromophore ($\tau_F$), which ranges from 0.5 to 20 ns for common organic fluorophores, reflects the average amount of time spent in the excited state before photon emission. As apparent from the Jablonski diagram (figure 1.1), the energy of the emitted photon is lower than the excitation energy. The Franck–Condon state relaxes to a lower vibronic level, leading to emission from a thermally equilibrated excited state, frequently to a higher vibrational level of the ground state ($S_0$). The resulting energetic difference between the excitation and emission maxima is known as the Stokes shift.

**Figure 1.1** Simplified Jablonski diagram
Steady-state fluorescence spectroscopy is the most commonly used technique to measure emission spectra in a laboratory. Although this method is somewhat limited in its scope, it can provide a myriad of useful data with relatively simple benchtop equipment. Steady-state fluorimeters include a light source to provide a constant monochromatic photon flux for sample excitation. Photons emitted are typically detected orthogonally to the excitation source. Correlating the number of photons emitted vs. wavelength (or energy) yields an emission spectrum that reveals the emission maxima and their relative intensities.

1.3 – Fluorescent Nucleoside Analogs

Because the naturally occurring, common nucleosides (A, G, T, U and C; figure 1.2) are practically non-emissive,⁴ oligonucleotides may be selectively modified with fluorescent nucleoside analogs. Ideally, the replacement nucleosides, while providing a spectroscopic handle, must not significantly perturb the native folding, dynamic and recognition features of the system under study. In addition, the photophysical characteristics of the implemented probes need to be sensitive to changes in their microenvironment to be able to serve as singular responsive probes. Although diverse emissive nucleoside analogs have been made over the years, few can be considered non-perturbing.¹ Perhaps the best known isomorphic analog is 2-aminopurine (2AP), a UV emitting isomer of A (figure 1.3a).¹³ This prototypical analog has been implemented in countless biophysical assays, illustrating the remarkable utility of isomorphic nucleoside analogs.¹⁴-¹⁶ To generate useful analogs of all letters of the genetic alphabet and across a variety of excitation and emission wavelengths,
we have initiated a program aimed at the design and implementation of novel emissive nucleosides, nucleotides and oligonucleotides.

Figure 1.2 Naturally occurring common ribonucleosides

![Chemical structures of nucleosides and nucleotides](image-url)
In designing new fluorescent nucleosides, several principles have been developed and employed. First, analogs should retain the highest possible structural similarity to the native nucleosides, with special consideration given to the maintenance of canonical Watson-Crick hydrogen-bonding faces. Second, the nucleoside analog should possess an absorption spectrum distinct from the native nucleosides (preferably red-shifted) to enable a selective excitation of the modified analog in the presence of the overwhelming majority native nucleotides. Third, the quantum efficiency of the modified nucleoside must be adequate for detection by
bench-top fluorescence spectrometers. Additionally, the fluorescent nucleoside analogs should exhibit long wavelength emission, ideally in the visible range. Finally, to be implemented as singular probes, the fluorescence emission of the analogs should be sensitive to changes in their microenvironment. These design criteria have led to the development of several useful fluorescent probes and, in turn, biophysical assays.

The first generation of fluorescent nucleoside analogs developed in our program was based upon conjugating aromatic five-membered rings to the 5-position of the pyrimidines (figure 1.3b). These nucleosides were synthesized with ease and incorporated into oligonucleotides enzymatically or through standard solid phase protocols. Although they possess modest quantum yields, these nucleoside analogs display fluorescence in the visible range, relatively large Stokes shifts, and are quite responsive. Related nucleosides with a fused ring system were also explored (figure 1.3c). The photohysical characteristics were found to depend on the nature of the fused heterocycle and its fusion position. In keeping with previously established design principles, we have also developed a family of nucleosides based on a quinazoline core (figure 1.4). This heterocyclic core was decorated with electron rich groups in positions either ortho or para to the pyrimidine’s C4 carbonyl, to potentially enhance polarization in the excited state. These nucleosides display a range of emission maxima and have proven to be useful when applied alone or as members of FRET pairs. Table 1.1 lists selected emissive nucleosides and their photophysical characteristics.
Figure 1.4 Quinazoline-based nucleoside family; R = D-ribose.
Table 1.1 Spectroscopic Properties of Isomorphic Fluorescent Nucleosides<sup>a</sup>

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AP</td>
<td>303</td>
<td>370</td>
<td>0.68</td>
</tr>
<tr>
<td>1</td>
<td>315</td>
<td>439</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>316</td>
<td>440</td>
<td>0.04</td>
</tr>
<tr>
<td>3</td>
<td>310</td>
<td>443</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>304</td>
<td>412</td>
<td>0.48</td>
</tr>
<tr>
<td>5</td>
<td>292</td>
<td>351</td>
<td>0.06</td>
</tr>
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<td>6</td>
<td>307</td>
<td>371</td>
<td>0.31</td>
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<td>358</td>
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<td>305</td>
<td>357</td>
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<td>10</td>
<td>324</td>
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<td>$\leq 0.01$</td>
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<td>11</td>
<td>320</td>
<td>395</td>
<td>0.16</td>
</tr>
<tr>
<td>12</td>
<td>349</td>
<td>440</td>
<td>0.42</td>
</tr>
</tbody>
</table>

<sup>a</sup>All values are for the free nucleoside in water. Values for $\lambda_{\text{abs}}$ are the local intensity maxima for the most red-shifted absorbance band. Values for $\lambda_{\text{em}}$ are the overall emission intensity maxima when excited at $\lambda_{\text{abs}}$. All quantum yields are relative.<sup>1, 6-8, 27</sup>

1.4 – Aims of the thesis

Thiophene-modified nucleoside 1 has a number of favorable photophysical properties. However, the low quantum yield (0.02) leaves room for improvement. The free rotation of the thiophene moiety about the single bond provides an effective means for non-radiative decay from the excited state. Hampering the rotation of the
thiophene moiety in viscous media leads to a dramatic increase in fluorescence intensity.\textsuperscript{28} Based on this, two approaches to improving the photophysical attributes of nucleoside 1 for potential biophysical assays were developed. First, incorporating multiple identical fluorescent nucleoside analogs into a single oligonucleotide can lead to alignment of the thiophene moiety either with itself or with adjacent native nucleobases, which may increase fluorescence quantum yield and sensitivity. Second, forming a covalent bond between the 3-position of the thiophene moiety and the pyrimidine leads to an expanded pi-system (figure 1.5a), along with a vast increase in quantum yield. A cytidine analog was synthesized to maintain a proper Watson-Crick hydrogen bonding face (figure 1.5b). In order to add to the growing toolbox of fluorescent nucleoside analog sets, multiple incorporations of a single nucleoside were explored and four novel fluorescent nucleoside analogs were synthesized and photophysically evaluated. Finally, the boundaries of current nucleoside analog utility in protein assays were pushed, resulting in an enhanced knowledge of the limits as well as advantages of isomorphic nucleoside analog FRET pairs.
Figure 1.5 Fused thiophene-modified nucleoside a) Proposed structure to limit the free rotation of the thiophene moiety; b) proper Watson–Crick base pairing between the modified nucleoside and native G ($R_1 = \text{D-ribose or 2'-deoxyribose}$).

1.5 – References


Chapter 1 contains sections of text and figures that are reprinted from: Noé, M. S.; Xie, Y.; Tor, Y. In *Methods for Studying Nucleic Acid/Drug Interactions*; Wanunu, M.; Tor, Y. Ed.; CRC Press (Taylor & Francis Group): Boca Raton, FL, 2012. The dissertation author is the main author for this work.
CHAPTER 2: Multiple Incorporations of Thiophene-modified Fluorescent Nucleosides into Oligodeoxynucleotides

Abstract. Incorporating multiple fluorescent nucleoside analogs into single oligodeoxynucleotides results in novel properties and enhanced fluorescence signal. With minimal impact upon duplex stability and structure, oligonucleotides containing three isomorphic fluorescent nucleosides (5-(thien-2-yl)-2'-deoxythymidine) display an enhanced on-signal for duplex annealing, melting, and mismatch detection. Depending on the location of the three modified nucleosides within the oligonucleotide sequence, the desired signal may be obtained for a specific assay. Placing the three nucleosides in adjacent positions results in a quenching of fluorescence emission, especially in a more structurally constrained duplex. This can be advantageous in displaying an increase in emission upon introduction of structural perturbations such as a mismatch base-pair or duplex melting. Placing the modified nucleosides in positions alternating with a native adenine residue results in an increase in fluorescence emission. This emission becomes more pronounced when the oligonucleotide is paired with a complementary strand to form a perfect duplex. This allows for detection of duplex formation with select complementary sequences.

2.1 – Introduction

As the fields of biology and chemistry progress and continue to tweeze apart the intricate roles of nucleic acids in the cell, fluorescence spectroscopy has proved
an invaluable tool. The demand for innovative fluorescent probes increases steadily along with the availability of nucleic acid assays, diagnostics, and drugs. By chemically introducing a minimal structural perturbation to native non-fluorescent nucleic acids, isomorphic fluorescent nucleoside analogs have been designed and implemented in biophysical assays to monitor events such as drug binding,\textsuperscript{1-5} RNA folding and cleavage,\textsuperscript{6-8} and RNA–protein interactions.\textsuperscript{9-10} The desired signal visualization and assay conditions dictate whether isomorphic nucleosides are required to display sensitivity to the microenvironment or lack thereof.\textsuperscript{11-12} Fluorescent nucleosides that are sensitive to their microenvironment can provide information about local parameters such as mismatch base pairing,\textsuperscript{13-16} the presence of oxidized bases,\textsuperscript{13} and pH.\textsuperscript{16} As the microenvironment changes, fluorescent nucleoside analogs may display wavelength shifts or changes in intensity. Additionally, nucleoside analogs may display sensitivity to other chromophores and fluorophores through ground-state and excited-state interactions. By placing multiple fluorescent nucleoside analogs into single oligonucleotides, these chromophoric interactions can be exploited to elicit enhanced fluorescence signal responses to biophysical events.

2.2 – Previous studies with multiply-modified oligonucleotides

Other laboratories have explored systems with multiple incorporations of chromophores and fluorophores into oligonucleotides. A majority of these studies involved probes having significant structural modifications compared to native nucleic acids and even abandon hydrogen-bonding faces altogether.\textsuperscript{17-19} The Kool laboratory developed a system in which a combination of a variety of chromophores with large pi-stacking surfaces tethered was together by a DNA backbone. This produced an
array of fluorescent dyes across the entire visible spectrum. The modifications result in bright fluorophores with interesting photophysical properties but move away from compatibility with biological systems, especially with regard to nucleic acids studies. Moving closer to isomorphicity, the Wagenknecht laboratories incorporated 5-pyrenyl-2'-deoxythymidine into oligonucleotides. Incorporation of this modified nucleoside into five adjacent positions at the center of an oligonucleotide resulted in excimer formation and a wavelength shift depending on the sequence of the complementary strand. A wavelength shift of the emission presented an unambiguous signal, but this system could only faithfully differentiate between five adjacent adenine and five adjacent guanine residues, which was accompanied by significant fluorescence emission quenching. The Fiebig laboratory studied oligonucleotides with multiple incorporations of isomorphic nucleoside analogue 2-aminopurine to explore the electronic properties of a DNA duplex. The resulting exciton and exciplex formation were observed through detailed analysis of changes in the absorption, excitation and emission spectra obtained through steady-state spectroscopy. While this study employed isomorphic nucleoside analogues, it was used as a platform to predict the electronic behavior of a DNA duplex rather than explore the development of biophysical assays.

2.3 – Previous studies with fused and tethered 5-modified fluorescent nucleoside analogs

Our program has focused on synthetic modifications of nucleosides that tether or fuse aromatic rings and heterocycles to the native nucleosides, especially the pyrimidines (figure 2.1). For example, placing a furan or thiophene moiety on
the 5-position of uridine (figure 2.1c) leads to highly sensitive nucleoside analogs, which have been used to detect abasic sites,\textsuperscript{13} monitor RNA–drug interactions,\textsuperscript{22} and measure DNA groove polarity.\textsuperscript{25} The single covalent bond linking the furan or thiophene heterocycle to the pyrimidine allows for free rotation, which imparts a molecular rotor behavior to the fluorophore.\textsuperscript{26} In viscous media and at low temperatures, the free rotation about this single bond is hampered, which results in an increase in fluorescence intensity. Structural analysis indicates that moieties linked to the 5-position of pyrimidines will be projected into the major groove of the B-form DNA duplex. Based on ease of synthesis and initial photophysical studies (figure 2.2), nucleoside 1 was chosen as a synthetically accessible, sensitive, and compatible probe. Nucleoside 1 has a red-shifted absorbance band at 314 nm and an emission maximum of 438 nm in water. Additionally, this modified fluorescent nucleoside analog displays sensitivity to solvent polarity (figure 2.2b), as demonstrated by the hypsochromic shift in fluorescence emission in the less polar environment of dioxane. Finally, nucleoside 1 does not display any significant fluorescence quenching in the presence of any of the native nucleosides as demonstrated by the Stern-Volmer plot (figure 2.2c). In conclusion, upon incorporation of 1 into an oligonucleotide, the thiophene moiety will be projected into the major groove of duplex DNA and display fluorescence sensitivity to its microenvironment, including the presence of adjacent chromophores and fluorophores.
Figure 2.1 Structure of isomorphic fluorescent nucleoside analogs made through the fusion of methoxybenzene (a) or thiophene (b) to a uridine core and through a single covalent linkage to the 5-position (c).

Figure 2.2 Modified nucleoside 1 summary. a) Structure and photophysical properties of 1; b) excitation (dashed) and emission (solid) spectra of 1 in water (blue), dioxane (black), and mixtures thereof (grey); c) Stern-Volmer plot of the fluorescence intensity of 1 in the presence of increasing concentrations of each native nucleotide (AMP, dTMP, GMP, and CMP) in water.

2.4 – System design

To further explore chromophore–chromophore interactions within a DNA duplex and the impact of multichromophoric modifications, three oligonucleotides were designed and synthesized. Each 17mer oligonucleotide includes either one or three incorporations of isomorphic fluorescent nucleoside 1 (figure 2.3). In designing
these three modified oligonucleotides, the highest possible sequence homology within the series was maintained. Oligonucleotide 2 includes a single incorporation of the fluorescent nucleoside analog 1 near the center of the sequence and serves as a baseline reference for emission intensity and behavior. Oligonucleotide 3 includes three total incorporations of the fluorescent nucleoside analog 1 in central positions of the sequence, each 1 alternating with dA residues. Oligonucleotide 4 contains three adjacent incorporations of nucleoside 1. All sequences were designed to contain a significant number of dG and dC residues near the termini to maintain a high thermodynamic stability of duplexes formed by hybridization to their perfect complements (5 and 6).

```
2  5' CCG GGA TA1 ATA GGC AG 3'
3  5' CCG GGA 1A1 A1A GGC AG 3'
4  5' CCG GGA A11 1AA GGC AG 3'
5  5' CTG CCT ATA TAT CCC GG 3'
6  5' CTG CCT TAA ATA CCC GG 3'
```

**Figure 2.3** Sequences of modified oligonucleotides 2–4 and their native perfect complements 5 and 6.

### 2.5 – Oligonucleotide synthesis

Standard solid-phase chemistry was used to synthesize modified oligonucleotides 2–4. This required synthesis of a 5'-protected phosphoramidite 9 (scheme 2.1). Compound 1 was prepared through a standard palladium-mediated cross-coupling reaction between commercially available 5-iodo-2'-deoxyuridine 7 and 2-(tributylstannyl)thiophene. The 5'-OH of the resulting modified nucleoside was
protected under standard conditions with 4,4'-dimethoxytrityl chloride (DMTrCl) and triethylamine in pyridine. Finally, phosphoramidite 9 was prepared by reacting the 5'-protected nucleoside 8 with 2-cyanoethyl N,N,N',N'-tetraisopropylphosphordiamidite in the presence of 1H-tetrazole. This phosphoramidite was diluted with anhydrous acetonitrile and placed directly onto a standard, automated DNA synthesizer. The resulting oligonucleotides were cleaved, deprotected, gel-purified, and de-salted using standard protocols. The oligonucleotides where characterized by MALDI-TOF mass spectrometry and detailed photophysical analysis.

Scheme 2.1 Synthesis of phosphoramidite 9. a) 2-(tributylstannyl)thiophene, PdCl₂(PPh₃)₂, N,N-dimethylformamide, 79%; b) 4,4'-dimethoxytrityl chloride, triethylamine, pyridine, 85%; (iPr₂N)₂POCH₂CH₂CN, 1H-tetrazole, acetonitrile, 63%.

2.6 – Initial photophysics and duplex stabilities

The fluorescence emission spectra of each oligonucleotide as a single strand and in a perfect duplex show clear contrasts in the photophysical behavior of each system (figure 2.4a). All oligonucleotides display an emission maximum near 438 nm, with no significant wavelength shift from the individual nucleoside 1. The fluorescence emission of singly modified oligonucleotide 2 is slightly quenched by
duplex formation (2•5), which is quite common with fluorescent nucleoside analogs. Interestingly, modified oligonucleotide 3, with three alternating incorporations of nucleoside 1, has nearly the same fluorescence intensity as oligonucleotide 2. Without taking neighboring effects into account, one would expect the fluorescence of 3 to have three times the emission intensity of 2, as it contains three times as many fluorophores. Upon hybridization with its native perfect complement (3•5), the fluorescence emission dramatically increases. However, the emission intensity is still less than three times that of duplex 2•5. Notably, both sequences of 2 and 3 have adenine residues surrounding the modified nucleoside. However, upon hybridization, the fluorescence emission behaves in opposite fashion for each strand (2•5 vs 3•5). This behavior suggests that pi-stacking of nucleoside 1 with native adenine residues does not lead to an increase in fluorescence on its own, but may lead to sensitivity to adjacent chromophores as well as those in distal positions.
In stark contrast, oligonucleotide 4, with three adjacent incorporations of nucleoside 1, has significantly lower fluorescence emission in the single strand. This low intensity is diminished even further upon hybridization (4•6), which suggests a self-quenching mechanism between the adjacent modified nucleosides. The adjacent nucleosides appear to impart an increase in duplex stability compared to the other modified oligonucleotides, as seen in the thermal denaturation curves (figure 2.4b). This is most likely due to pi-stacking of the thiophene moieties in the major groove of the duplex. Additionally, the thermal denaturation curves establish proper duplex formation for all modified oligonucleotides.

**Figure 2.4** a) Fluorescence emission of oligonucleotides 2 (blue), 3 (red), and 4 (green) in single strand (dashed) and duplex (solid); b) thermal denaturation curves of duplexes 2•5 (blue), 3•5 (red), and 4•6 (green); c) average melting point (Tm) for each duplex in phosphate buffer (100mM NaCl, 10mM phosphate, pH 7).

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<td>3•5</td>
<td>57</td>
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<td>61</td>
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</table>
2.7 – Structural studies of modified duplexes

To confirm double helix formation of modified oligonucleotide duplexes 2•5, 3•5, and 4•6, circular dichroism spectroscopy was used to study the secondary structure. All modified oligonucleotides displayed characteristics of B-form DNA helices (figure 2.5), with only slight deviation from the native control duplexes. Of note is the added signal in duplex 4•6 centered around 320 nm (figure 2.5c) near the red-shifted absorbance band present in the absorption spectrum (figure 2.6). This most likely indicates an alignment of the adjacent thiophene moieties along the major groove of the helix, which is in contrast to duplex 3•5 where the fluorescent nucleosides are positioned so that such an alignment is not possible (figure 2.3 and 2.6b).
Figure 2.5 CD spectra of (a) 2·5 (blue), (b) 3·5 (red), (c) 4·6 (green), and (d) all spectra overlayed along with a native control duplex (black) in phosphate buffer (100mM NaCl, 10mM phosphate, pH 7).

Figure 2.6 a) Absorption spectra of prefect duplexes of 2·5 (blue), 3·5 (red), and 4·6 (green) in phosphate buffer (100mM NaCl, 10mM phosphate, pH 7); b) schematic representations of duplexes of 2·5 (blue), 3·5 (red), and 4·6 (green), where X represents nucleoside 1.
2.7 – On-signal for duplex annealing and melting

In fluorescence spectroscopy, both increases and decreases in fluorescence intensity may be effectively used as signals to follow a biophysical assay. However, as systems become increasingly complex, decreases in fluorescence signal may be less reliable. This is a consequence of the fact that any number of molecules and phenomena may cause fluorescence quenching, leading to a false signal. In contrast, increases in fluorescence emission, referred to as an “on-signal”, are rarely due to such side events. As such, it is advantageous to design assays to produce an on-signal to indicate the progress of a biophysical event such as drug binding, duplex melting, and duplex annealing.

As seen in figure 2.7, when duplex 4•6 separates, the fluorescence intensity increases. This increase in fluorescence can be used to follow strand separation in real time. Additionally, upon hybridization of 3, duplex 3•5 exhibits a large increase in fluorescence intensity, allowing for real time monitoring of duplex formation. Although this requires the presence of a 5'-TATATAT-3' -sequence motif, this probe could still prove to have great utility. Additionally, oligonucleotide 3 can be annealed to a shorter complementary strand (15mer) containing only a 5'-TATA-3' -sequence motif (oligonucleotide 10), commonly encountered in genomes of all species, which results in an even stronger on-signal (figure 2.8).
Figure 2.7 a) Absorption and emission spectra of single stranded (dashed) and perfect duplexes (solid) of 3 (red) and 4 (green) in phosphate buffer (100mM NaCl, 10mM phosphate, pH 7); b) schematic representation of on-signals for annealing of 3 to 3•5 and melting of 4•6 to 4, where X indicates the position of nucleoside 1.

Figure 2.8 a) Emission spectra of 3 (red dashed) and duplexes 3•5 (red solid) and 3•10 (black solid) in phosphate buffer (500mM NaCl, 10mM phosphate, pH 7); b) sequence of native oligonucleotide 10; c) schematic representation of duplexes 3•10 (black) (does not represent actual bulge structure) and 3•5 (red) where X indicates nucleoside 1. [Note that fluorimeter slit widths were decreased to 3.5 nm to accommodate large signal increases]
2.8 – Bulge formation to attempt to disrupt electrostatic interactions of adjacent thiophene moieties in oligonucleotide 4

As indicated by the CD spectrum (figure 2.5c) and the low fluorescence of duplex 4-6, the adjacent thiophene moieties interact in a manner that decreases fluorescence intensity. In order to diminish these interactions, oligonucleotide 4 (a 17mer) was annealed to oligonucleotide 11, a 16mer with a single dT nucleotide missing from the middle position of the sequence (see figure 2.9a) in hopes of forming a small bulge in the duplex. Interestingly, the fluorescence intensity was not greatly affected by this structural perturbation (figure 2.9b). The proper formation of a duplex was confirmed through thermal denaturation studies (figure 2.9c).
**Figure 2.9** a) Sequence of oligonucleotide 11, where the dT residue adjacent to the deleted dT is highlighted in pink; b) Fluorescence emission of oligonucleotide 4 (green dashed), perfect duplex 4·6 (green solid), and bulge duplex 4·11 (pink); c) thermal denaturation curves of duplexes 4·6 (green) and 4·11 (pink); d) average melting point (T_m) for each duplex in phosphate buffer (500mM NaCl, 10mM phosphate, pH 7).

2.9 – Mismatch dC detection with oligonucleotide 4

In addition to allowing for real-time detection of nucleic acid dynamics such as melting and annealing, fluorescent nucleoside analogues can be used to detect single nucleotide polymorphisms (SNPs). In addition to detecting abasic sites,13 fluorescent nucleoside analogues can detect C mismatches14 and G mismatches.15 In a screen of all single mismatches (dG, dT, and dC), the largest signal enhancement obtained from a single mismatch resulted from hybridizing 4 with oligonucleotide 12 (figure 2.10a). This duplex contains a cytidine in place of the center adenine residue that
should base pair with the central modified nucleoside in oligonucleotide 4. Notably, even though there is an increase in fluorescence compared to perfect duplex 4•6, the fluorescence intensity of the mismatch duplex 4•12 is still lower than that of the single strand 4. This leads to the possibility of an ambiguous signal in potential biophysical assays, especially if all duplexes are not fully hybridized.

a) 12 5’ CTG CCT TAC ATA CCC GG 3’

Figure 2.10 a) Sequence of mismatch dC oligonucleotide 12 where the mismatch dC residue is highlighted in purple; b) fluorescence emission of oligonucleotide 4 (green dashed), perfect duplex 4•6 (green solid), and mismatch dC duplex 4•12 (purple) in phosphate buffer (500mM NaCl, 10mM phosphate, pH 7).

2.10 – Enhanced mismatch dC detection with oligonucleotide 4

To obtain an enhanced, unambiguous on-signal for a single mismatch, concepts from sections 2.8 and 2.9 were combined. In order to break up potential self-quenching between the adjacent modified nucleosides in oligonucleotide 4 and introduce a single mismatch cytidine, oligonucleotide 13 (a 16mer) was designed
Additionally, oligonucleotide 14, analogous to 13 but with a single thymidine mismatch was employed to confirm the selectivity of the mismatch dC signal. Duplex 4•14 failed to produce a fluorescence signal intensity above that of the single strand 4 (not shown). However, duplex 4•13 displayed the largest observed fluorescence intensity increase, which was distinctly higher than that of single strand 4 (figure 2.11b). Thermal denaturation curves confirmed proper hybridization of all duplexes (figure 2.11c) and indicated that 4•13 was the least stable.

![Figure 2.11](image)

**Figure 2.11** a) Sequence of oligonucleotides 11, 13, and 14; b) fluorescence emission of oligonucleotide 4 (green dashed), bulge duplex 4•11 (pink solid), and mismatch dC bulge duplex 4•13 (cyan); c) thermal denaturation curves of duplexes 4•6 (green), 4•11 (pink), and 4•13 (cyan); d) average melting point (Tₘ) for each duplex in phosphate buffer (500mM NaCl, 10mM phosphate, pH 7).
2.11 – Enhanced mismatch dC detection structural confirmation

To confirm proper helix formation of modified oligonucleotide 4·13, circular dichroism spectroscopy was used to study the secondary structure. Even with a slight bulge in the duplex due to hybridizing a 17mer with a 16mer as well as a mismatch dC, duplex 4·13 displayed a signature CD signal similar to a B-form DNA helix (figure 2.12). The extra signal centered near 320 nm displayed by perfect duplex 4·6 is still present in duplex 4·13 but to a lesser degree. This indicates a decrease in structural alignment of the chromophores in the adjacent modified nucleoside analogues, which can help, in part, to explain the increase in fluorescence emission of 4·13.

Figure 2.12 CD spectra of 4·6 (green), 4·13 (cyan) and a native control duplex (black) in phosphate buffer (100mM NaCl, 10mM phosphate, pH 7).
2.11 – Concluding remarks

These studies successfully demonstrated the utility of multiply-modified oligonucleotides in several model systems. The enhanced fluorescence signal obtained from these oligonucleotides may prove useful in biophysical assays for a number of reasons. Instead of adding a large, structurally perturbing fluorophore to obtain a higher quantum yield, several modified isomorphic analogs may be employed. Not only do these nucleosides allow for more biologically accurate secondary structures, they may also be compatible with endogenous enzymes. As previously demonstrated, T7 RNA polymerase will faithfully incorporate 5-(thien-2-yl)-uridine into an oligonucleotide. Further investigations into enzyme tolerance of this 5-modified nucleoside could prove fruitful. For example, it could be determined if repair enzymes will be recruited to these modified sites within oligonucleotides and if their binding and activity could be monitored via fluorescence signal changes. Overall, this model system provides an interesting platform for increased fluorescence sensitivity in structurally unperturbed biophysical assays.

Appendix – Experimental information

A.1 – General procedures

Reagents were purchased from Sigma-Aldrich, Rasayan Inc. (Encinitas, CA), Acros, and VWR and were used without further purification unless otherwise specified. Solvents were purchased from Sigma-Aldrich and Fisher Scientific. Anhydrous acetonitrile was obtained from Glen Research (Sterling, VA). Anhydrous
N,N-dimethylformamide was obtained using a two-column purification system (Glasscontour System, Irvine, CA). NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Reactions were monitored with analytical thin-layer chromatography (TLC) performed on pre-coated silica gel aluminum-backed plates (Merck Kieselgel 60 F254). All experiments involving air and/or moisture sensitive compounds were carried out under an argon atmosphere. Column chromatography was performed with silica gel particle size 40-63 μm. NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Mass spectra were obtained on an Agilent 6230 HR-ESI-TOF MS at the Molecular Mass Spectrometry Facility at the UCSD Chemistry and Biochemistry Department. All absorption measurements were obtained at 21 °C on a Shimadzu UV 2450 absorption spectrometer using a quartz cuvette with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany). Steady state fluorescence measurements were obtained at 21 °C on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer using a quartz fluorescence cell with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany). All CD spectra were recorded on an Aviv 215 Circular Dichroism Spectrometer in a 350 μL quartz cell with a path length of 0.1 cm at 25 °C.

A.2 – Synthetic procedures

5-(thien-2-yl)-2'-deoxythymidine (1). A flask containing 5-iodo-2'-deoxyuridine (7) (3.50 g, 9.88 mmol) and PdCl₂(PPh₃)₂ (347 mg, 0.495 mmol) dissolved in anhydrous DMF (100 mL) was heated while stirring to 80 °C. To this mixture, 2-tributylstannyl thiophene (4.70 mL, 14.8 mmol) was added via syringe and allowed to stir at 80 °C
overnight until the flask contained an even black coating around a clear brown solution. The reaction mixture was removed from heated and reduced to a thick brown oil under reduced pressure. This oil was dissolved in MeCN (~50 mL) and rinsed five times with ample quantities of hexanes to help remove toxic tin compounds. The acetonitrile was removed under reduced pressure to produce a brown foam. The product was purified by silica column chromatography (9/1 DCM/acetonitrile) and recrystallized from hot methanol to produce lovely white crystals (1.90 g, 6.13 mmol, 62% yield). $^1$H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 11.67 (s br, 1H), 8.57 (s, 1H), 7.45–7.44 (d, $J$=5.0 Hz, 1H), 7.39–7.38 (d, $J$=3.6 Hz, 1H), 7.04–7.02 (t, $J$=4.3 Hz, 1H), 6.22–6.19 (t, $J$=6.3 Hz, 1H), 5.31–5.26 (m, 2H), 4.31–4.30 (m, 1H), 3.83 (m, 1H), 3.71–3.61 (m, 2H), 2.26–2.14 (m, 2H); $^{13}$C NMR (DMSO-d$_6$, 100 MHz): $\delta$ 162.0, 150.0, 136.4, 134.7, 127.1, 126.4, 123.2, 123.0, 109.0, 88.3, 85.5, 70.7, 73.1, 61.5, 41.1; HRMS: [M + Na]$^+$ calculated for $C_{13}H_{14}N_2O_5SNa^+$, 333.0516; found, 333.0514.

5-(thien-2-yl)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine (8). A flask containing 1 (423 mg, 1.4 mmol) and 4,4'-dimethoxytrityl chloride (546 mg, 1.61 mmol) was dissolved in anhydrous pyridine (14 mL) and allowed to stir at room temperature under argon. To this mixture, triethylamine (0.29 mL, 2.1 mmol) was added via syringe and allowed to stir overnight at room temperature. The reaction mixture was condensed to an orange oil under reduced pressure. The product was purified by silica column chromatography (95/4/1 DCM/methanol/triethylamine) to produce a peach foam (727 mg, 1.19 mmol, 85% yield). $^1$H NMR (CDCl$_3$, 400 MHz): matched to published data; $^{28}$ ESI-MS: [M + Na]$^+$ calculated for $C_{34}H_{32}N_2O_7SNa^+$, 635.18; found, 635.14.
5-(thien-2-yl)-3'-(2-cyanoethyldiisopropylphosphoramidite)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine (9). A flask containing 8 (727 mg, 1.19 mmol) was dissolved in anhydrous acetonitrile (21.2 mL) and stirred at room temperature. To this mixture, 2-cyanoethyl N,N,N',N'-tetraisopropylphosphoramidite (415 μL, 1.31 mmol) was added via syringe and allowed to stir under argon. To this mixture, 1H-tetrazole (2.64 mL, 0.45 M in acetonitrile, 1.19 mmol) was added dropwise via syringe over thirty minutes. The reaction mixture was stirred at room temperature for 4 hours, monitoring closely by TLC (DCM/MeOH 9/1). The reaction was quenched with a few drops of methanol and dried to a white foam under reduced pressure. The product was purified by flash silica column chromatography (98/1/1 Chloroform/MeOH/triethylamine) to produce a mixture of diastereomers as a bright white foam (610 mg, 0.75 mmol, 63% yield). 1H NMR (CDCl₃, 400 MHz): δ 9.83 (s br, 1H), 7.85 (s, 1H), 7.40–7.38 (d, J=7.3 Hz, 2H), 7.27–7.12 (m, 7H), 6.81–6.80 (d, J=3.6 Hz, 1H), 6.76–6.74 (d, J=8.8 Hz, 4H), 6.69–6.66 (t, J= 4.4 Hz, 1H), 6.43–6.39 (m, 1H), 4.62–4.58 (m, 1H), 4.20–4.19 (m, 1H), 3.84–3.79 (m, 1H), 3.72 (s, 6H), 3.58–3.52 (m, 2H), 3.42–3.39 (m, 1H), 3.33–3.29 (m, 1H), 2.64–2.59 (m, 4H), 2.31–2.24 (m, 1H), 1.27–1.24 (m, 1H), 1.16–1.15 (d, J=6.8 Hz, 12H); 31P NMR (CDCl₃, 162 MHz): δ 150.0, 149.6 (H₃PO₄ reference); ESI-MS: [M + Na]⁺ calculated for C₄₃H₄₉N₄O₈PSNa⁺, 835.29; found, 835.35.

A.3 – Oligonucleotide synthesis and purification

All modified and native oligonucleotides were synthesized on an Expedite 8909 DNA synthesizer at 1.0 μmole scale (500 Å CPG). Phosphoramidite 9 was
dissolved in anhydrous acetonitrile at a concentration of 100 mg/mL and placed directly onto a port on the DNA synthesizer. All standard phosphoramidites (5’-dimethoxytrityl-N-benzoyl-2’-deoxyadenosine-3’-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite, 5’-dimethoxytrityl-N-benzoyl-2’-deoxycytidine-3’-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite, 5’-dimethoxytrityl-N-isobutyryl-2’-deoxyguanosine-3’-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite, and 5’-dimethoxytrityl-2’-deoxythymidine-3’-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite) were purchased from Glen Research (Sterling, VA) and dissolved in anhydrous acetonitrile to prepare solutions at 25 mg/mL. The coupling time for phosphoramidite 9 was increased to 253 seconds, and all other couplings were performed via standard, trityl-off procedures, including standard capping, coupling, oxidizing, and deprotection solutions (Glen Research).

Columns containing CPG-oligonucleotides were removed from the DNA synthesizer, and any residual solvent was removed under reduced pressure. The plastic columns containing the solid CPG support were cut open and the contents were placed into 5mL reaction vessels. To each vial, 3mL of 30% aqueous ammonium hydroxide was added and the vessel was sealed and placed at 55 °C overnight. After allowing the reaction vessels to cool to room temperature, excess ammonia was removed under a steady stream of argon gas. The remaining water was lyophilized and the samples were resuspended in 150 μL or less of 7M urea in 1X TBE buffer. Samples were loaded onto a pre-heated 20% polyacrilamide gel and run at roughly 300 mM for 6 hr. Resulting UV-active bands were excised from the gel and the oligonucleotides were extracted into 4.0 mL 0.5 NaOAc buffer (pH 7.4) at room temperature overnight. Samples were de-salted by loading oligonucleotides
onto Waters C-18 Sep-Pak columns, rinsing with water, and eluting with 40% acetonitrile in water. Samples containing oligonucleotides (determined by UV-vis profile) were lyophilized and dissolved in 1 mL deionized water. Oligonucleotides were quantified based on absorption at 260 nm and the calculated (nearest neighbor method) molar extinction coefficient (http://www.ambion.com/techlib/misc/oligo_calculator.html). MALDI-TOF spectra were obtained in 3-hydroxypicolinic acid matrix with ammonium citrate buffer, after mixing with ion-exchange resin (PE biosystems), using a 25mer DNA standard for calibration. Spectra were obtained on a PE Biosystems Voyager-DE STR MALDI-TOF spectrometer in positive-ion mode. See table A.1 for all calculated and found masses.

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</table>

A.4 – Oligonucleotide hybridizations

All oligonucleotide samples were prepared in 5 μM solutions of either 100 mM or 500 mM NaCl (as indicated) in 10 mM sodium phosphate buffer (pH 7.0). Samples were heated to 95 °C for 5 minutes and cooled to room temperature over 2–3 hrs. Samples were then placed at 0 °C until spectroscopic measurements were obtained.
UV-Vis spectra were obtained on a Shimadzu UV 2450 absorption spectrometer using a 125 μL quartz cuvette with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany) at 21 °C. Steady-state fluorescence spectra were obtained on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer using a 125 μL quartz fluorescence cell with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany) at 21 °C with slit widths of 5 nm (unless otherwise noted). Thermal denaturation curves were obtained on a Beckman-Coulter DU® 640 spectrometer with a high performance temperature controller and a micro auto six sample holder. Samples were heated from room temperature to 80 °C at a rate of 0.5 °C min\(^{-1}\) with optical monitoring every minute at 260 nm. Beckman-Coulter software provided on the spectrometer determined the first derivative from the melting profile to calculate the inflection point/T\(_m\). For CD spectra, samples were prepared to contain 25 μM of the duplex in 100 mM NaCl, 10mM sodium phosphate buffer (pH 7).

2.12 – References


CHAPTER 3: Design, Synthesis and Spectroscopic Properties of Extended and Fused Pyrrolo-dC and Pyrrolo-C Analogs

Abstract. The syntheses of four fluorescent nucleoside analogs, related to pyrrolo-C (PyC) and pyrrolo-dC (PydC) through the conjugation or fusion of a thiophene moiety, are described. A thorough photophysical analysis of the nucleosides, in comparison to PyC, is reported.

3.1 – Introduction

Fluorescence spectroscopy is a powerful tool for the detailed investigation of nucleic acids and their diverse biomolecular interactions, due to its sensitivity and molecular specificity. These features become accessible when suitable fluorescent probes exist. The insignificant emission of the native nucleobases presents unique opportunities for the design of non-perturbing fluorophores. Creative modifications with minimal structural perturbations have resulted in the development of isomorphic fluorescent nucleoside analogs. These fluorophores have been successfully employed in the monitoring of real-time biochemical events such as drug binding, RNA folding and cleavage, and RNA–protein interactions. The specific utility of a fluorescent nucleoside depends on its photophysical behavior under the desired assay conditions. Analogs that display sensitivity to their microenvironment, through wavelength shifts or changes in intensity, can provide information about local parameters such as polarity, viscosity, and pH. Equally, analogs with minimal sensitivity to the microenvironment exhibiting desired absorption and emission bands
may be utilized in a FRET-based system. The photophysical properties of any given analog cannot be predicted through simple structural analysis of the fluorophore. Only upon the synthesis of a probe and analysis of its photophysical characteristics can its properties, such as quantum yield, Stokes shift, brightness and sensitivity to polarity, be determined to indicate its most apt implementation.

Pyrrolo-dC (PydC) and pyrrolo-C (PyC) are structurally modified fluorescent deoxycytidine and cytidine analogs, which maintain a proper Watson–Crick H-bonding face (Figure 1). A nominal structural modification leads to a fluorophore with a significant quantum yield and an absorbance band, which is red-shifted from those of the native nucleosides and aromatic amino acid residues. The quantum yield of PyC decreases upon incorporation into oligonucleotides and is quenched even further upon duplex formation. Still, PyC has been used in numerous biophysical assays including spectroscopic visualization of the elongation complex of an RNA polymerase and the monitoring of RNA-folding dynamics. While modifications of PyC have gained popularity in recent years, new analogs may expand the structural repertoire and diversify the photophysical properties attainable to facilitate the development of novel assays.

Our program has focused on the development of diverse non-perturbing fluorescent nucleoside analogs via the conjugation or fusion of aromatic heterocycles to the native bases, especially the pyrimidines. For example, placing a furan or thiophene moiety on the 5-position of uridine leads to sensitive nucleoside analogs, which have been used to detect abasic sites, oxidatively damaged nucleosides, and monitor RNA–drug interactions. Although these useful nucleosides possess measurable sensitivity, low fluorescence quantum yields leave room for
improvement. Studies have demonstrated that hampering the free rotation of the furan or thiophene moieties in viscous media dramatically increases the quantum yield. This inspired the design and synthesis of nucleoside analogs 1–4 (figure 3.1). In correlation to our 5-modified pyrimidines, analogs 1 and 2 represent a PyC core, which is extended via conjugation to a thiophene. Analogs 3 and 4, in contrast, represent a fused system, similar to both PyC and our previously reported nucleoside alphabet, in which the chromophore possesses no rotatable bonds. As such, deoxy- and ribonucleosides 1–4 can be viewed as new PyC analogs. We report the synthesis and evaluation of these analogs, as well as compare and contrast their photophysical features with one another and with PydC and PyC.

Figure 3.1. Watson-Crick hydrogen-bonding faces of C and PyC. Extended (1 and 2) and fused (3 and 4) PyC analogs.

3.2 – Synthesis

The syntheses of compounds 1–4 were based upon the implementation of a palladium-mediated cross-coupling reaction followed by a crucial intramolecular cyclization step (Schemes 1 and 2). This approach employs native nucleosides as starting materials, eliminating ambiguities commonly associated with glycosylation
reactions regarding the isolation of the correct anomer and regioisomer. Previously reported syntheses of PyC and its analogs were based upon the one-pot Sonogashira-coupling and ensuing cyclization through the use of palladium and copper catalysts. This cross-coupling reaction was performed successfully between 5-iodouridine and a variety of substituted alkynes.\textsuperscript{26,32-33} The resulting furanopyrimidines were fluorescent but lacked a proper Watson-Crick hydrogen-bonding face. Following solid-phase incorporation into oligonucleotides, ammonolysis resulted in the efficient conversion of the furanopyrimidines into cytidine analogs.\textsuperscript{34} This conversion may also be achieved by treating the furanopyrimidines with methanolic ammonia before incorporation into oligonucleotides.

\begin{center}
\textbf{Scheme 3.1.} Synthesis of extended analogs 1 and 2
\end{center}
The initial synthetic attempts to obtain extended thiophene analogs 1 and 2 were based upon this approach. Unfortunately, all efforts to convert the furanopyrimidines resulted in complex mixtures and low yields of the desired cytidine analogs. An alternate synthesis was attempted (Scheme 3.1) using 5-iodo-2′-deoxycytidine (5) and cytidine as starting materials. Cytidine (6) was acetylated and iodinated using established methods,\(^{35}\) while compound 5 was purchased from a commercial source. Compounds 5 and 7 reacted readily with 2-ethynylthiophene under standard cross-coupling conditions, but the included copper catalyst failed to facilitate further cyclization. Compounds 8 and 9 were, therefore, isolated, purified, and screened for an alternate metal-based cyclization catalyst. Gold catalysts activate alkyne moieties to a greater degree than other metal ions,\(^{36}\) so a sodium tetrachloroaurate(III) dihydrate was employed based upon successful cyclization of related heterocycles.\(^{37}\) Although the cyclization yields were not optimal, this concise synthesis produced compound 1 in an overall yield of 41% in 2 steps and compound 2 in an overall yield of 13% in 4 total steps.

The syntheses of the fused thiophene PyC analogs 3 and 4 (Scheme 3.2) were initiated with the use of commercially available 11 and known modifications of uridine to afford 13.\(^{38}\) Cytidine starting materials were explored, but all reactions involving cytidine analogs were lower yielding and more problematic to purify. As numerous methods exist for the exchange of a C-4 carbonyl for an amine, the conversion of a uridine to a cytidine core was left until the penultimate step. The palladium-mediated Stille coupling reaction was high yielding for both the unprotected
2'-deoxynucleoside 11 and the acetylated ribonucleoside 13 and afforded mildly fluorescent nucleosides 14 and 16. Installing a halogen at the 3-position of the thiophene allowed for the screening of catalysts capable of inducing the desired intramolecular cyclization to the pyrrole moiety. Since the 5-position of the thiophene undergoes electrophilic aromatic substitution more readily than the 3-position, an excess of bromine was added to afford dibrominated products 17 and 18. Selective zinc-mediated debromination of the 5-position yielded compounds 19 and 20, respectively. Following the activation of the C-4 carbonyl, a one-pot conversion of the U to C analog and O-deacetylation produced compounds 21 and 22.
Scheme 3.2. Synthesis of fused analogs 3 and 4

The intramolecular cyclization of 21 and 22 to yield the desired fused PyC analogs proved to be the biggest synthetic hurdle. Buchwald–Hartwig amination reactions were first attempted through the use of various ligands and palladium catalysts. All reactions yielded, however, only starting material and/or the
debrominated products 5-(thiophen-2-yl)-cytidine or 5-(thiophen-2-yl)-2'-deoxycytidine. Copper catalysts with various ligands were explored next. Of all attempted reactions, only a single set of conditions which include copper(I)iodide, cesium carbonate, and $N,N'$-dimethylethylenediamine ($N,N'$-DMEDA) provided the cyclized products 3 and 4.\(^{42}\) Although the cyclization reactions were not as high yielding as the preceding steps, they provided ample quantities of nucleosides 3 and 4 for full analytical and photophysical studies. Compound 3 was synthesized in an overall yield of 18\% over 6 steps, while compound 4 was prepared in an overall yield of 22\% over 6 steps.

### 3.3 – Photophysics

The photophysical properties of nucleosides 1–4 along with PyC and PydC are summarized in Table 3.1. As expected, the presence of a ribose or deoxyribose moiety (1 vs. 2, or 3 vs. 4) had little impact on the photophysics of the respective chromophores. The nucleosides displayed red-shifted absorption bands at 369 nm (1), 371 nm (2), and 357 nm (3 and 4); longer wavelengths than the parent PydC (343 nm) and PyC (342 nm) chromophores in water (see figure 3.2A). These bands were further red-shifted when the nucleosides were dissolved in dioxane, an aprotic, less polar solvent. Somewhat surprisingly, in aqueous solution, the thiophene extended analogs displayed only slight shifts in emission maxima, 469 nm (1) and 473 nm (2), from the parent nucleosides PydC (463 nm) and PyC (461 nm). However, the significantly higher quantum yields of 0.41 and 0.43 for 1 and 2, respectively, are over nine times that of PyC (0.04) and PydC (0.05). The quantum yields of 1 and 2 showed moderate sensitivity to solvent polarity, exhibiting a hypsochromic shift and increase
of fluorescence in dioxane. The exceptional property of nucleosides 1 and 2 is the brightness value ($\Phi\varepsilon$), which is primarily due to large extinction coefficient ($\varepsilon$) values of 11.6 and $12.9 \times 10^3$ M$^{-1}$ cm$^{-1}$ (1 and 2, respectively, in water). Both nucleosides show a linear correlation between Stokes shift and solvent polarity with reasonable polarity sensitivity values (see appendix). Notably, both the absorption and emission spectra show sensitivity to solvent polarity for the entire nucleoside series.

**Table 3.1 Photophysical data for PydC, PyC and nucleosides 1–4**

<table>
<thead>
<tr>
<th>compound</th>
<th>solvent</th>
<th>$\lambda_{\text{abs}}$ (nm)$^a$</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\varepsilon^b$</th>
<th>$\Phi^c$</th>
<th>Stokes shift$^d$</th>
<th>brightness ($\Phi\varepsilon$)</th>
<th>polarity sensitivity$^e$</th>
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<tbody>
<tr>
<td>PydC</td>
<td>H$_2$O</td>
<td>342</td>
<td>461</td>
<td>4.03</td>
<td>0.05</td>
<td>7.6</td>
<td>0.20</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>351</td>
<td>439</td>
<td>5.00</td>
<td>0.10</td>
<td>5.7</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>PyC</td>
<td>H$_2$O</td>
<td>343</td>
<td>463</td>
<td>5.04</td>
<td>0.04</td>
<td>7.6</td>
<td>0.20</td>
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<tr>
<td></td>
<td>Dioxane</td>
<td>349</td>
<td>439</td>
<td>5.00</td>
<td>0.10</td>
<td>6.0</td>
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<tr>
<td>1</td>
<td>H$_2$O</td>
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<td>5.7</td>
<td>4.8</td>
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<td>Dioxane</td>
<td>383</td>
<td>449</td>
<td>13.6</td>
<td>0.50</td>
<td>3.9</td>
<td>6.8</td>
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<td>12.9</td>
<td>0.43</td>
<td>5.9</td>
<td>5.5</td>
<td>73</td>
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<td>0.47</td>
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<td>474</td>
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<tr>
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<td>Dioxane</td>
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<td>0.74</td>
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<td></td>
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</table>

$^a$ Long $\lambda$ maximum. $^b$ $10^3$ M$^{-1}$cm$^{-1}$. $^c$ Relative quantum yields. $^d$ $10^3$ cm$^{-1}$. $^e$ Polarity sensitivity values are expressed in cm$^{-1}$/(kcal·mol$^{-1}$) and represent the slope of the line in the plot of stokes shift versus solvent polarity (see appendix for details)
The fused thiophene PyC analogs exhibited photophysical characteristics that were distinctive from the thiophene extended system (see figure 3.2B). In water, nucleosides 3 and 4 exhibited the most red-shifted emission maxima (474 and 478 nm, respectively) along with the lowest emission intensity of this nucleoside series. In dioxane, the increased emission intensity is dwarfing in comparison to that in water, with maxima at 447 nm. While the Stokes shifts of nucleosides 3 ($7.0 \times 10^3 \text{ cm}^{-1}$) and 4 ($7.1 \times 10^3 \text{ cm}^{-1}$) were comparable to the parent PydC and PyC ($7.6 \times 10^3 \text{ cm}^{-1}$), they were higher than 1 ($5.7 \times 10^3 \text{ cm}^{-1}$) and 2 ($5.9 \times 10^3 \text{ cm}^{-1}$). Most striking is the extreme sensitivity that the quantum yields of 3 and 4 exhibited to solvent polarity. In the polar protic environment of water, 3 and 4 both displayed a very low quantum yield of 0.01. However, in an aprotic and less polar environment, the quantum yields of 3 and 4 increased dramatically. These quantum yield values of 0.70 and above are exceptional, considering the minimal structural modification from the virtually non-emissive cytidine nucleosides. This increase occurs concomitantly with a decrease in Stokes shift resulting in values of approximately $5 \times 10^3 \text{ cm}^{-1}$. Nucleosides 3 and 4 have similar $\varepsilon$ in both water (2.38 and $2.42 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and dioxane (3.16 and $3.21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), which are significantly lower than those of nucleosides 1 and 2. This results in comparatively lower brightness values for nucleosides 3 (0.024 in water and 2.2 in dioxane) and 4 (0.024 in water and 2.4 in dioxane). Notably, the brightness values for 3 and 4 are nearly 100-fold higher in dioxane than in water.
In summary, the PyC family is joined by new members. The effective syntheses of nucleosides 1–4 via novel, metal-catalyzed cyclization reactions resulted in four nucleosides displaying complementary photophysical properties. These nucleosides make a significant and diverse addition to the growing toolbox of non-perturbing, isomorphic, fluorescent analogs.

**Figure 3.2.** Absorption (dashed) and emission (solid) spectra of 2 (A) and 4 (B) in water (blue) and dioxane (black).
Appendix – Supporting information

A.1 – Materials and synthesis

A.1.1 – General Procedures

Reagents were purchased from Sigma-Aldrich, Rasayan Inc. (Encinitas, CA), Acros and VWR, and were used without further purification unless otherwise specified. Solvents were purchased from Sigma-Aldrich and Fisher Scientific. Anhydrous $N,N$-dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained using a two-column purification system (Glasscontour System, Irvine, CA). NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Reactions were monitored with analytical thin-layer chromatography (TLC) performed on pre-coated silica gel aluminum-backed plates (Merck Kieselgel 60 F254). All experiments involving air and/or moisture sensitive compounds were carried out under an argon atmosphere. Column chromatography was performed with silica gel particle size 40–63 μm. NMR spectra were obtained on Varian Mercury 400 MHz and Jeol ECA 500 MHz spectrometers. Mass spectra were obtained on an Agilent 6230 HR-ESI-TOF MS at the Molecular Mass Spectrometry Facility at the UCSD Chemistry and Biochemistry Department.

A.1.2 – Synthesis

Synthesis of 2-ethynylthiophene via desilylation of 2-((Trimethylsilyl)-ethynyl)thiophene was reported elsewhere.$^{43}$ Syntheses of compounds $7,^{35} 13,^{38} 14,^{39} 17,^{40}$ and $19^{41}$ were previously reported.
5-(2-Ethynylthiophene)-2′-deoxycytidine (8). A solid mixture of 5-iodo-2′-
deoxyctydine (5) (5.00 g, 14.1 mmol), Pd(dppf)Cl₂ (413 mg, 0.565 mmol), and
copper(I) iodide (134 mg, 0.704 mmol) was placed in a flask. To the degassed flask
containing the solids, was added previously purged (30 mins each) anhydrous DMF
(47mL) and anhydrous triethylamine (7mL) via syringe. The flask was heated to 40°C
and the orange suspension was allowed to stir for a few minutes. To the warmed
suspension was added a previously purged (30 mins purging under argon) 2-
ethynylthiophene (3.00 g, 27.8 mmol) in triethylamine (7mL) via syringe, as the
reaction mixture turned yellow. The heat was increased to 50°C and reaction was
allowed to stir for 3 hours with monitoring by TLC (9/1 DCM/MeOH). Upon
completion, the orange-tan colored reaction mixture was removed from heat and
allowed to cool to room temperature. Solvent volume was reduced to ca.1/3 of original
amount under vacuum followed by dilution with DCM. The flask was placed on an ice
bath to induce voluminous precipitation. The white solid was filtered off, and the
supernatant was subjected to further rounds of precipitaion. The crude was
combined and recrystallized in methanol to afford light-tan colored crystals of 8 (4.03
g, 12.1 mmol, 85%). ¹H NMR (DMSO-d₆, 400 MHz): δ 8.28 (s, 1H), 7.74 (s br,
1H(NH)), 7.62–7.60 (d, J= 5.0 Hz, 1H), 7.41–7.40 (d,J= 3.5 Hz, 1H), 7.09–7.07 (m,
1H), 7.02 (s br, 1H(NH)), 6.12–6.09 (t, J=6.5 Hz, 1H), 5.22–5.21 (d, J=4.2 Hz, 1H,
OH), 5.10–5.08 (t, J=5.0 Hz, 1H, OH), 4.22–4.18 (m, 1H), 3.79–3.78 (d, 1H), 3.64–
3.52 (m, 2H), 2.18–2.12 (m, 1H), 2.04–1.97 (m, 1H); ¹³C NMR (DMSO-d₆, 125 MHz):
δ 164.2, 154.0, 145.5, 133.5, 129.0, 128.0, 122.6, 90.0, 88.0, 87.0, 86.0, 85.8, 70.8,
61.6, 41.5; HRMS: [M + Na]⁺ calculated for C₁₅H₁₅N₃O₄SNa⁺, 356.0675; found, 356.0677.
Extended (thien-2-yl)-pyrrolo-2'-deoxycytidine (1). A solution of 8 (3.00 g, 9.00 mmol) in ethanol (55mL) was heated to 45 °C. To the warmed solution was added a solution of NaAu(Cl₄)•2H₂O (71 mg, 0.18 mmol) in ethanol (5mL). Reaction temperature was raised to 55–60 °C and allowed to reflux overnight. The reaction was monitored by TLC (97.5/2.5 MeCN/NH₄OH (aq)) and was stopped prematurely to minimize deglycosylation of cyclized product. After allowing the reaction mixture to cool, the solvent was removed under reduced pressure. The crude mixture was purified by silica column chromatography (98/2 MeCN/NH₄OH (aq)) to afford product 1 as a yellow solid (1.44 g, 4.32 mmol, 48%, not based on recovered starting material). ¹H NMR (DMSO-d₆, 500 MHz): δ 11.85 (s, 1H), 8.61 (s, 1H), 7.56–7.54 (d, \(J=5.5\) Hz, 1H), 7.53–7.52 (d, \(J=3.5\) Hz, 1H), 7.11–7.09 (m, 1H), 6.43 (s, 1H), 6.22–6.20 (t, \(J=6.5\) Hz, 1H), 5.26–5.25 (d, \(J=4.5\) Hz, 1H, OH), 5.11–5.09 (t, \(J=5.3\) Hz, 1H, OH), 4.23–4.20 (m, 1H), 3.88–3.86 (m, 1H), 3.67–3.57 (m, 2H), 2.35–2.31 (m, 1H), 2.02–1.97 (m, 1H); ¹³C NMR (DMSO-d₆, 100 MHz): δ 160.4, 154.5, 136.8, 134.7, 134.5, 128.8, 127.1, 125.7, 109.7, 97.1, 88.6, 87.7, 70.6, 61.6, 42.1; HRMS: [M + Na]⁺ calculated for C₁₅H₁₅N₃O₄SNa⁺, 356.0675; found, 356.0677.

5-(2-Ethynylthiophene)-2',3',5'-tri-O-acetyl-cytidine (9). A solid mixture of 7 (2.20 g, 4.43 mmol), Pd(dppf)Cl₂ (163 mg, 0.223 mmol), and copper(I) iodide (48 mg, 0.252 mmol) was placed in a flask. To the degassed flask was added anhydrous DMF (15 mL) and anhydrous triethylamine (4.4 mL) via syringe. To the suspension was added 2-ethynylthiophene (1.35g, 12.5 mmol) in triethylamine (1mL) and anhydrous DMF (3 mL) via syringe as the reaction mixture turned yellow. The flask was allowed to stir overnight at room temperature with monitoring by TLC (95/5 DCM/MeOH).
Upon completion, the orange-tan colored reaction mixture was diluted with cold methanol (20–30 mL) and allowed to stir to induce precipitation. The suspension was filtered and the supernatant was reduced in volume under vacuum. Further amounts of cold methanol were added to induce additional precipitation followed by filtration. The crude precipitate was combined and recrystallized in methanol to afford white crystals of 9 (1.65g, 3.47 mmol, 78%). $^1$H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 8.16 (s, 1H), 7.99 (s br, 1H, NH), 7.63–7.62 (m, 1H), 7.43–7.42 (m, 1H), 7.31 (s br, 1H, NH), 7.10–7.08 (m, 1H), 5.87–5.86 (d, $J$=4.2 Hz, 1H), 5.46–5.44 (m, 1H), 5.35–5.32 (t, $J$=6.2 Hz, 1H), 4.37–4.32 (m, 1H), 4.26–4.20 (m, 2H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H); $^{13}$C NMR (DMSO-d$_6$, 100 MHz): $\delta$ 170.7, 170.1, 170.0, 164.5, 153.7, 146.9, 133.7, 129.6, 128.2, 122.5, 91.0, 90.3, 88.1, 85.0, 79.6, 73.3, 70.2, 63.5, 21.3, 20.99, 20.97; HRMS: [M + Na]$^+$ calculated for C$_{21}$H$_{21}$N$_3$O$_8$SNa$^+$, 498.0942; found, 498.0943.

Extended (thien-2-yl)-pyrrolocytidine (2). A solution of 9 (1.65 g, 3.46 mmol) in ethanol (21mL) was heated to 45 °C. To the warmed solution was added a solution of NaAu(Cl$_4$)$\cdot$2H$_2$O (27 mg, 0.068 mmol) in ethanol (2mL). Reaction temperature was raised to 70 °C, to encourage dissolution, and refluxed for > 24 hours. Reaction was monitored by TLC (99/1 MeCN/NH$_4$OH (aq)). During the course of the reaction, the gold catalyst also promoted minimal deacetylation, so small amounts of gold catalyst (5mg) were added to encourage progression of cyclization. Reaction was eventually stopped prematurely to prevent any metal catalyzed deglycosylation of cyclized product. Mixture was allowed to cool to room temperature, then solvent volume was reduced by $\frac{1}{2}$ and diluted with dichloromethane (10mL) to encourage partial gravity filtration of gold-catalyst. To the mixture was added aqueous NH$_4$OH (37%, 2 – 3 mL)
and let stir for 30-40 mins at room temperature. After complete deacetylation, residual ammonia gas was bubbled out before the remaining solvents were removed under vacuum. The yellow crude was purified by silica column chromatography (98/2 DCM/MeOH) to afford 2 as a yellow solid (414 mg, 1.18 mmol, 34%, not based on recovered starting material). $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 11.87 (s, 1H, NH), 8.76 (s, 1H), 7.57–7.55 (m, 2H), 7.13–7.11 (m, 1H), 6.42 (s, 1H), 5.92 (d, $J$=1.8 Hz, 1H), 5.51–5.50 (d, $J$=4.1 Hz, 1H, OH), 5.25–5.22 (t, $J$=5.0, 1H, OH), 5.03–5.02 (d, $J$=5.2 Hz, 1H, OH), 4.00–3.99 (m, 2H), 3.95–3.93 (m, 1H), 3.82–3.77 (m, 1H), 3.67–3.62 (m, 1H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz): $\delta$ 160.4, 154.8, 137.2, 134.9, 134.4, 128.9, 127.2, 125.8, 109.8, 97.0, 91.8, 84.8, 75.7, 69.2, 60.6; HRMS: [M + Na]$^+$ calculated for C$_{16}$H$_{15}$N$_3$O$_5$SNa$^+$, 372.0625; found, 372.0625.

5-(Thien-2-yl)-2′,3′,5′-O-acetyl-uridine (16). A flask containing 5-iodo-2′,3′,5′-O-acetyl-uridine (13) (2.13 g, 4.29 mmol) and PdCl$_2$(PPh$_3$)$_2$ (151 mg, 0.214 mmol) dissolved in anhydrous DMF (43 mL) was heated while stirring to 80 °C. To this mixture, 2-tributylstannyl thiophene (1.57 mL, 4.93 mmol) was added via syringe and allowed to stir at 80 °C overnight until the flask contained an even black coating around a clear brown solution. The reaction mixture was removed from heated and reduced to a thick brown oil under reduced pressure. This oil was dissolved in MeCN (~40 mL) and rinsed five times with ample quantities of hexanes to help remove toxic tin compounds. The acetonitrile was removed under reduced pressure to produce a brown foam. The product was purified by silica column chromatography (9/1 DCM/acetone) and recrystallized from hot methanol to produce lovely white crystals (1.63 g, 3.60 mmol, 84% yield). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 9.11 (s br, 1H), 7.75 (s,
1H), 7.43–7.42 (d, J=3.6 Hz, 1H), 7.31-7.30 (d, J=5.1 Hz, 1H), 7.06–7.03 (t, J=4.3 Hz, 1H), 6.17–6.16 (d, J=5.5 Hz, 1H), 5.41–5.35 (m, 2H), 4.46–4.35 (m, 3H), 2.14 (s, 3H), 2.11 (s, 3H), 2.08 (s, 3H); 13C NMR (CDCl₃, 100 MHz): δ 170.4, 169.9, 160.9, 149.6, 134.3, 133.1, 127.4, 126.1, 125.5, 111.4, 94.5, 87.3, 80.6, 73.1, 70.6, 63.5, 21.0, 20.8, 20.7; HRMS: [M + Na]⁺ calculated for C₁₉H₂₀N₂O₉SNa⁺, 475.0782; found, 475.0780.

5-(3,5-Dibromothien-2-yl)-2′,3′,5′-O-acetyl-uridine (18). Bromine (0.37 mL, 7.1 mmol) in carbon tetrachloride (8.9 mL) was added dropwise over 30 minutes to solution of 16 (1.40g, 3.09 mmol) in 1,2-dichloroethane (44 mL) stirring at 0 °C. The solution was allowed to stir for an additional hour at 0 °C before quenching with DCM. The organic layer was washed with saturated NaHCO₃, brine, 5% NaHSO₃, and water, repeating as necessary until it has no remaining yellow color. The organic layer was dried over Na₂SO₄, filtered, and dried to an off-white foam under reduced pressure. The product was purified by silica column chromatography (9/1 DCM/acetone) to produce a white foam (1.74 g, 2.85 mmol, 92% yield). 1H NMR (CDCl₃, 400 MHz): δ 9.00 (s br, 1H), 8.13 (s, 1H), 7.00 (s, 1H), 6.20–6.18 (m, 1H), 5.39–5.35 (m, 2H), 4.41–4.38 (m, 1H), 4.35–4.34 (m, 2H), 2.14 (s, 3H), 2.12 (s, 3H), 2.00 (s, 3H); 13C NMR (CDCl₃, 100 MHz): δ 170.4, 169.9, 169.8, 160.9, 149.3, 138.8, 132.4, 129.3, 115.1, 108.5, 108.4, 87.3, 80.3, 73.3, 70.3, 63.4, 20.9, 20.8, 20.7; HRMS: [M + Na]⁺ calculated for C₁₉H₁₈Br₂N₂O₉SNa⁺, 630.8892; found, 630.8890.

5-(3-Bromothien-2-yl)-2′,3′,5′-O-acetyl-uridine (20). A mixture of solid 18 (1.70 g, 2.80 mmol) and zinc dust (454 mg, 6.95 mmol) was suspended in anhydrous DMF (35 mL). To this suspension acetic acid (0.30 mL, 5.00 mmol) and acetic anhydride
(0.13 mL, 1.4 mmol) were added via syringe. The reaction was heated to 80 °C and stirred for 4 hrs, until small, round beads formed at the bottom of the clear yellow solution and no starting material remained (monitored by TLC). The reaction mixture was filtered through a cotton plug and the solvent was removed under reduced pressure to produce a yellow oil. The product was purified by silica column chromatography (92/8 DCM/acetone) and recrystallized from methanol and ethyl acetate to produce white crystals (1.22 g, 2.30 mmol, 82% yield). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 8.77 (s br, 1H), 7.42–7.40 (d, $J$=5.6 Hz, 1H), 7.04–7.02 (d, $J$=5.6 Hz, 1H), 6.23–6.20 (m, 1H), 5.41–5.37 (m, 2H), 4.41–4.38 (m, 1H), 4.34–4.33 (m, 2H), 2.14 (s, 3H), 2.13 (s, 3H), 1.94 (s, 3H); $^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$170.4, 169.9, 169.8, 160.9, 149.4, 139.1, 130.4, 127.8, 127.7, 109.9, 108.9, 87.1, 80.3, 73.3, 70.3, 63.4, 20.77, 20.74, 20.68; HRMS: [M + Na]$^+$ calculated for C$_{19}$H$_{19}$BrN$_2$O$_9$SNa$^+$, 552.9887; found, 552.9886.

5-(3-Bromothien-2-yl)-2'-deoxycytidine (21). A mixture of solid 19 (221 mg, 0.467 mmol) and p-toluenesulfonyl chloride (178 mg, 0.933 mmol) was dissolved in anhydrous acetonitrile (15.6 mL). Anhydrous triethylamine (0.2 mL, 1.4 mmol) was added and the reaction was allowed to stir at room temperature overnight. The reaction was monitored by TLC (9/1 DCM/acetone) until no starting material was visible. Reaction was placed directly into a pressure tube to which 37% aqueous NH$_4$OH (~15 mL) was added. The reaction was heated to 40 °C, stirring for roughly 72 hours, then concentrated to dryness under reduced pressure. Off-white solid was purified by silica column chromatography (9/1 DCM/MeOH) to produce a white, powdery solid (153 mg, 0.394 mmol, 84% yield). $^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 8.20
(s, 1H), 7.60–7.59 (d, J=5.2 Hz, 1H), 7.13–7.12 (d, J=5.2 Hz, 1H), 6.27–6.24 (t, J=6.0 Hz, 1H), 4.38–4.35 (m, 1H), 3.95–3.92 (m, 1H), 3.79–3.66 (m, 2H), 2.44–2.39 (m, 1H), 2.22–2.16 (m, 1H); \(^{13}\)C NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 164.2, 156.4, 143.5, 130.9, 128.9, 128.2, 113.1, 99.9, 87.8, 86.7, 70.5, 61.2, 41.3; HRMS: [M + Na]\(^+\) calculated for C\(_{13}\)H\(_{14}\)BrN\(_3\)O\(_4\)SNa\(^+\), 409.9781; found, 409.9783.

5-(3-Bromothien-2-yl)-cytidine (22). A mixture of solid 20 (495 mg, 0.931 mmol) and p-toluenesulfonyl chloride (355 mg, 1.86 mmol) was dissolved in anhydrous acetonitrile (31 mL). Anhydrous triethylamine was added and the reaction was allowed to stir at room temperature overnight. The reaction was monitored by TLC (9/1 DCM/acetone) until no starting material was visible. Reaction was placed directly into a pressure tube to which 37% aqueous NH\(_4\)OH (~30 mL) was added. The reaction was heated to 40 °C, stirring for roughly 72 hours, then concentrated to dryness under reduced pressure. Off-white solid was purified by silica column chromatography (9/1 DCM/MeOH) to produce a white, powdery solid (294 mg, 0.726 mmol, 78% yield). \(^1\)H NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 8.30 (s, 1H), 7.61–7.60 (d, J=5.4 Hz, 1H), 7.13–7.12 (d, J=5.4 Hz, 1H), 5.90–5.89 (d, J=2.9 Hz, 1H), 4.20–4.15 (m, 2H), 4.05–4.02 (m, 1H), 3.88–3.84 (m, 1H), 3.73–3.69 (m, 1H); \(^{13}\)C NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 164.2, 156.6, 143.8, 130.8, 128.8, 128.2, 122.3, 113.1, 91.2, 84.6, 75.3, 69.2, 60.2; HRMS: [M + Na]\(^+\) calculated for C\(_{13}\)H\(_{14}\)BrN\(_3\)O\(_5\)SNa\(^+\), 425.9730; found, 425.9728.

Fused-thieno-[2,3-d]-pyrrolo-2'-deoxycytidine (3). A mixture of solid 21 (150 mg, 0.386 mmol), cesium carbonate (252 mg, 0.772 mmol), and copper(I) iodide (7 mg, 0.0386 mmol) was dissolved in anhydrous DMF (13 mL). \(N,N'\)-
dimethylethylenediamine (8uL, 0.08 mmol) was added and the reaction was allowed to stir at 60 °C overnight as it turned from blue to green to light brown. The reaction was monitored by TLC (98/2 MeCN/NH₄OH (aq)) as a fluorescent green spot appeared along with the disappearance of the starting material. The reaction was cooled to room temperature and several drops of saturated EDTA solution were added. The reaction was evaporated to dryness under reduced pressure, resulting in a brown solid. This mixture was purified by silica column chromatography (98/2 MeCN/NH₄OH (aq)) to produce a light yellow solid (45 mg, 0.147 mmol, 37% yield).

¹H NMR (CD₃OD, 400 MHz): δ 9.02 (s, 1H), 7.50–7.49 (d, J=5.2 Hz, 1H), 7.06–7.05 (d, J=5.2 Hz, 1H), 6.41–6.38 (t, J=6.1 Hz, 1H), 4.44–4.40 (m, 1H), 4.05–4.02 (m, 1H), 3.95–3.91 (m, 1H), 3.84–3.80 (m, 1H), 2.60–2.54 (m, 1H), 2.24–2.18 (m, 1H); ¹³C NMR (CD₃OD, 125 MHz): δ 162.7, 155.3, 144.3, 134.1, 128.1, 114.2, 112.0, 105.2, 88.0, 87.9, 70.0, 61.0, 41.7; HRMS: [M + Na]⁺ calculated for C₁₃H₁₃N₃O₄SNa, 330.0519; found, 330.0523.

_Fused-thieno-[2,3-d]-pyrrolo-cytidine (4)._ A mixture of solid 22 (100 mg, 0.247 mmol), cesium carbonate (161 mg, 0.494 mmol), and copper(I) iodide (355 mg, 1.86 mmol) was dissolved in anhydrous DMF (13 mL). N,N'-dimethylethylenediamine (8uL, 0.14 mmol) was added and the reaction was allowed to stir at 60 °C overnight as it turned from blue to green to light brown. The reaction was monitored by TLC (97/3 MeCN/NH₄OH (aq)) as a fluorescent green spot appeared along with the disappearance of the starting material. The reaction was cooled to room temperature and several drops of saturated EDTA solution were added. The reaction was evaporated to dryness under reduced pressure, resulting in a brown solid. This
mixture was purified by silica column chromatography (97/3 MeCN/NH₄OH (aq)) to produce a light yellow solid (42 mg, 0.13 mmol, 52% yield). ¹H NMR (DMSO-d₆, 400 MHz): δ 11.74 (s br, 1H), 8.99 (s, 1H), 7.59–7.58 (d, J=5.1 Hz, 1H), 7.08–7.07 (d, J=5.1 Hz, 1H), 5.94 (s, 1H), 5.51 (s br, 1H), 5.31–5.30 (m, 1H), 5.05–5.04 (m, 1H), 4.12–3.94 (m, 3H), 3.84–3.81 (m, 1H), 3.67–3.64 (m, 1H); ¹³C NMR (DMSO-d₆, 100 MHz): δ 163.7, 154.5, 144.5, 135.5, 128.9, 113.9, 113.4, 104.4, 91.6, 84.7, 75.5, 69.1, 60.6; HRMS: [M – H]⁻ calculated for C₁₂H₁₂N₅O₅S⁻, 322.0503; found, 322.0502.

A.2 – Photophysical studies

A2.1 – Experimental details and calculations

PydC and PyC were obtained from Berry & Associates (Dexter, MI). Stock solutions of nucleosides were prepared by dissolving a known mass of the compound in spectrophotometric grade DMSO (Sigma Alrich). DMSO stock solutions were prepared in concentrations of 3.77 x 10⁻³ M, 3.55 x 10⁻³ M, 3.00 x 10⁻³ M, 2.86 x 10⁻³ M, 5.42 x 10⁻³ M, and 5.38 x 10⁻³ M for PydC, PyC, 1, 2, 3, and 4, respectively. Water-dioxane mixtures were prepared with spectrophotometric grade 1,4-dioxane and de-ionized water. A series of solutions containing 0, 10, 30, 70 and 100 v/v % water in dioxane were prepared. E₅(30) values for each solvent mixture was determined by dissolving a small amount of Reichardt’s dye in the solution and measuring the most red-shifted absorption maximum. This value was converted into E₅(30) using the formula: E₅(30) =28592/λₐ₅. All absorption measurements were obtained at 21 °C on a Shimadzu UV 2450 absorption spectrometer using a quartz cuvette with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany).
Steady state fluorescence measurements were obtained at 21 °C on a Jobin Yvon Horiba FLuoroMax-3 luminescence spectrometer using a 500 μL quartz fluorescence cell with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany). A variety of slit widths were used to obtain the full range of spectra for each nucleoside. Each spectroscopy sample was prepared by diluting the DMSO stock solutions so that each solution contained only 0.4 v/v % DMSO. Three replicates of each spectroscopy measurements were taken and demonstrated negligible error.

Extinction coefficients were determined by making Beer’s Law plots of concentration versus absorbance for each nucleoside at four or more different concentrations. Each Beer’s Law plot was determined three times for each nucleoside in each solvent, with negligible error. The relative quantum yields of each compound were determined using the formula:

\[ \Phi_u = \Phi_s \times \left( \frac{A_u}{A_s} \right) \times \left( \frac{I_u}{I_s} \right) \times \left( \frac{n_u^2}{n_s^2} \right), \]

where each absorbance (A) and integrated area of the emission spectrum (I) was measured in triplicate. For PydC and PyC, anthracene in ethanol (λ\text{abs} = 367, Φ = 0.27)\textsuperscript{45} was used as a standard. Additionally, the quantum yields of PydC and PyC in ethanol, 0.22 and 0.18, respectively, were determined. For compounds 1–4, coumarin 1 dissolved in acetonitrile (λ\text{abs} = 367, Φ = 1.03)\textsuperscript{46} was used as a standard. The refractive index of each solvent was obtained from The CRC Handbook of Chemistry and Physics (92nd ed.) 2011–2012.
A2.2 – Spectra and graphs

Figure A2.1 A) Absorption (dashed) and emission (solid) spectra of PydC in water (blue) and dioxane (black) and mixtures (grey). B) Linear relationship between stokes shift and solvent polarity ($E_T(30)$) of PydC in dioxane, water and mixtures.

Figure A2.2 A) Absorption (dashed) and emission (solid) spectra of PyC in water (blue) and dioxane (black) and mixtures (grey). B) Linear relationship between stokes shift and solvent polarity ($E_T(30)$) of PyC in dioxane, water and mixtures.
**Figure A2.3** A) Absorption (dashed) and emission (solid) spectra of 1 in water (blue) and dioxane (black) and mixtures thereof (grey). B) Linear relationship between stokes shift and solvent polarity ($E_r(30)$) of 1 in dioxane, water and their mixtures.

**Figure A2.4** A) Absorption (dashed) and emission (solid) spectra of 2 in water (blue) and dioxane (black) and mixtures thereof (grey). B) Linear relationship between stokes shift and solvent polarity ($E_r(30)$) of 2 in dioxane, water and their mixtures.
Figure A2.5 A) Absorption (dashed) and emission (solid) spectra of 3 in water (blue) and dioxane (black) and mixtures thereof (grey). B) Linear relationship between stokes shift and solvent polarity ($E_r(30)$) of 3 in dioxane, water and their mixtures. C) Relationship between emission intensity and solvent polarity of 3 in dioxane, water and their mixtures.
Figure A2.6 A) Absorption (dashed) and emission (solid) spectra of 4 in water (blue) and dioxane (black) and mixtures thereof (grey). B) Linear relationship between stokes shift and solvent polarity ($E_T(30)$) of 3 in dioxane, water and their mixtures. C) Relationship between emission intensity and solvent polarity of 3 in dioxane, water and their mixtures.

A.3 – NMR Spectra
Figure A3.1 $^1$H NMR of 8

Figure A3.2 $^{13}$C NMR of 8
Figure A3.3 $^1$H NMR of 1

Figure A3.4 $^{13}$C NMR of 1
Figure A3.5 $^1$H NMR of 9

Figure A3.6 $^{13}$C NMR of 9
Figure A3.7 $^1$H NMR of 2

Figure A3.8 $^{13}$C NMR of 2
Figure A3.9 $^1$H NMR of 16

Figure A3.10 $^{13}$C NMR of 16
Figure A3.11 $^1$H NMR of 18

Figure A3.12 $^1$H NMR of 18
Figure A3.13 $^1$H NMR of 20

Figure A3.14 $^{13}$C NMR of 20
Figure A3.15 $^1$H NMR of 21

Figure A3.16 $^{13}$C NMR of 21
**Figure A3.17** $^1$H NMR of 22

**Figure A3.18** $^{13}$C NMR of 22
**Figure A3.19** $^1$H NMR of 3

**Figure A3.20** $^{13}$C NMR of 3
Figure A3.21 $^1$H NMR of 4

Figure A3.22 $^1$H NMR of 4
Figure A3.23 $^{13}$C NMR of 4

3.4 – References


30. However, see references 17 and 31.


Chapter 3, in full, is a reprint of: Noé, M. S.; Ríos, A. C.; Tor, Y. Design, Synthesis and Spectroscopic Properties of Extended and Fused Pyrrolo-dC and Pyrrolo-C Analogs, Org. Lett. in press May 30, 2012. The dissertation author is the main author and researcher for this work.
CHAPTER 4: Fluorescent Nucleoside Analogs – Illuminating the Path into the Future

Abstract. The implementation of fluorescent nucleoside analogs into the mainstream world of enzyme assays faces several hurdles. The small, non-perturbing nature of many fluorescent nucleoside analogs makes them ideal candidates to be tolerated by any number of enzymes. This has the potential to enable detailed biophysical exploration of mechanism and dynamics in real-time. However, the small size and relatively small quantum yield of many fluorescent nucleoside analogs leads to a fluorescence sensitivity that may be hard to interpret and may be overwhelmed by the sheer number of fluorescent amino acid residues present in most enzymes. With careful planning, however, implementation of fluorescent nucleosides in any number of enzyme-based assays remains a promising possibility. In particular, lessons learned from attempts to monitor RNA helicase Ded1 activity in real-time via a fluorescent nucleoside FRET-pair can help guide future studies.

4.1 – Introduction

As the utility of fluorescent nucleoside analogs progresses, their implementation in more complex biophysical assays becomes more accessible. Moving towards an ultimate goal of cellular compatibility and tracking, it is essential to first establish compatibility of fluorescent nucleoside analogs with enzymes. Additionally, assays involving fluorescent nucleosides in nucleic acid systems and the enzymes that interact with them can lead to a detailed, real-time understanding of
particular molecular events. Fluorescent nucleoside analogs have been successfully employed in assays monitoring nucleic acid dynamics,\textsuperscript{1-2} RNA–drug interactions,\textsuperscript{3-5} as well as detection of single nucleotide polymorphisms.\textsuperscript{6-8} However, a vast majority of these assays take place in an isolated model system outside of the presence of full-length proteins and enzymes. These assays can be useful for screening large libraries of compounds and determining relative binding affinities of drugs, but often times these binding affinities do not translate effectively to actual biological systems. Additionally, indirect monitoring of protein activity has been accomplished through the use of fluorescent nucleoside analogs.\textsuperscript{9} A more fundamental application of fluorescent nucleoside analogs would be to monitor nucleic acid dynamics in the actual presence of endogenous enzymes. Previous studies have employed 2-aminopurine to monitor the processive activity of DNA helicases.\textsuperscript{10-11} Fluorescent nucleoside analog pyrrolo-dC has been employed to characterize the T7 RNA polymerase transcription bubble in elongation complexes.\textsuperscript{12} However, these studies involve the interpretation of a somewhat ambiguous change in fluorescence intensity of a single nucleoside analog. Other assays have employed large fluorophores to end-label oligonucleotides for FRET systems. This method of end-labeling can be used to measure a variety of enzyme activities.\textsuperscript{13-15} However, using end-labeling techniques can add significant structural perturbations as well as provide global rather than local information about nucleic acid dynamics. In order to overcome many of these disadvantages, an assay was designed to monitor the local melting of an RNA duplex in the presence of the non-processive RNA helicase DED1 through the use of two fluorescent nucleoside analogs in a FRET pair.
4.2 – RNA helicases and DED1 background

RNA helicases are ubiquitous proteins found in all three domains of life as well as many viruses. These enzymes bind to and remodel RNA and ribonucleoprotein complexes (RNPs), obtaining the energy to do so through ATP hydrolysis (for recent and concise reviews see references 16–18). Nearly every single aspect of RNA metabolism involves RNA helicases. RNA helicases are structurally related to DNA helicases and have been divided into superfamilies and families based on amino acid sequence and structure homology. The largest family of helicases contains an Asp-Glu-Ala-Asp (DEAD) sequence motif and is known as the DEAD box RNA helicase family. Found in all eukaryotes, DEAD box helicases have been associated with transcription, pre-mRNA splicing, ribosome biogenesis, translation, and RNA degradation. Interestingly, the molecular mechanism by which these helicases actually unwind and reorganize RNA structures has yet to be illuminated. Importantly, unlike well-known DNA helicases, DEAD box helicases show little or no processivity and appear to melt RNA duplexes in small non-sequence-specific regions. DED1 is a DEAD-box protein found in Saccharomyces cerevisiae and is highly conserved among eukaryotes. It is analogous to human DDX3 protein. DED1 has been shown to both unwind and anneal RNA duplexes. Unwinding activity occurs in an ATP-dependent fashion, while annealing occurs without ATP hydrolysis. As the DED1 gene has already been isolated, and S. cerevisiae is a readily available model organism, DED1 was chosen as a model ATP-dependent RNA helicase.
Most current methods of monitoring RNA unwinding and melting involve the use of radiolabeled RNA strands, quenching reactions at various time points, and visualizing gel shifts of single and double stranded RNA. The use of a fluorescent nucleoside analogue FRET pair in two complimentary RNA strands could enable the real-time visualization of local strand separation. This tool could become particularly powerful if utilized in a stopped-flow fluorescence spectrometer, revealing a previously unattained and detailed view of helicase activity.

4.3 – Basic resonance energy transfer background

Förster Resonance Energy Transfer (FRET) involves the nonradiative dipole–dipole interaction between two chromophores, commonly referred to as a donor and an acceptor. The transfer efficiency of a FRET pair can be predicted through analysis of their spectral properties. Specifically, the absorbance of the acceptor must sufficiently overlap with the emission of the donor. The overlap integral \( J(\lambda) \) is a function of the corrected fluorescence intensity of the donor with the total area under the curve that is normalized to unity \( (F_D(\lambda)) \) and the extinction coefficient of the acceptor \( (\varepsilon_A(\lambda)) \) all at wavelength \( \lambda \) (Eqn. 1).

\[
J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda
\]  

Eqn. 1

If the optical density of the acceptor is minimal at the wavelength of excitation of the donor, the sensitized emission of the acceptor can be selectively observed. The efficiency of energy transfer is distance-dependent, and the Förster critical radius \( (R_0) \) is the interchromophoric distance at which the transfer efficiency is 50% (20 to 60 Å for common FRET pairs). This critical distance is a function of the overlap integral
(\(J(\lambda)\)), the refractive index of the medium (\(n\)), the quantum yield of the donor (\(Q_D\)), and the relative orientation factor of the transition dipoles (\(\kappa^2\)) (Eqn. 2).

\[
R_o = 9.78 \times 10^3 (\kappa^2 n^{-4} Q_D J(\lambda))^{1/6}
\]

Eqn. 2

When FRET assemblies are studied in aqueous media and the fluorophores are freely rotating, the refractive index (\(n\)) is 1.33, and the relative orientation factor (\(\kappa^2\)) is assumed to be 2/3. This value reflects a dynamic random averaging of the donor and acceptor orientation. When the fluorophores are covalently linked in a manner that would impede free rotation, accurate estimation of the orientation factor may present challenges.

The FRET efficiency (\(E\)), also known as the energy transfer efficiency, can be determined from the ratio of the fluorescence intensity of the donor alone (\(F_D\)) and its intensity in the presence of the acceptor (\(F'_D\)) (Eqn. 3).

\[
E = 1 - \frac{F'_D}{F_D} = \frac{1}{1 + (r/R_o)^6}
\]

Eqn. 3

This ratio represents the fraction of photons absorbed by the donor that is responsible for energy transfer to the acceptor. As the resonance energy transfer efficiency is proportional to the inverse 6th power of the distance between the donor and acceptor, sensitized FRET data can provide exceptionally useful information in a variety of biochemical systems and biophysical assays.

4.4 – Fluorescent nucleoside analog FRET pair

In order to obtain data from the actual melting bubble of an RNA duplex within an RNA helicase, two isomorphic nucleoside analogs were selected to serve as a
FRET pair. A previous study implemented two fluorescent nucleosides as a FRET pair to measure distances within a DNA duplex. However, two isomorphic fluorescent nucleoside analogs have yet to be placed into an RNA double helix. For this purpose ribonucleoside 1 (7-methoxyquinazoline-2,4(1H,3H)-dione ribonucleoside), developed in the Tor laboratory, was chosen to be the FRET donor (figure 4.1a) and pyrrolo-cytidine (PyC) was chosen to function as the FRET acceptor (figure 4.1b). In water, nucleoside 1 has a red-shifted absorbance band near 300 nm, where PyC has a minimal optical density (figure 4.1c). Additionally, nucleoside 1 has a fluorescence emission maximum at 357 nm in water (Φ = 0.08), which overlaps nicely with the absorbance band of PyC (λ_{em} = 463 nm, Φ = 0.04, table 3.1). Based on the spectral overlap, the calculated Förster critical radius (R_0) for this FRET pair is 25 Å, which is ideal for a double-stranded oligoribonucleotide. Titrating acceptor PyC into a solution of donor nucleoside 1 effectively demonstrated the feasibility of this FRET pair (figure 4.2). As PyC was added to the solution, the emission of nucleoside 1 centered near 360 nm decreased, as the emission of PyC appeared near 460 nm.
Figure 4.1 Isomorphic fluorescent nucleoside analog FRET pair. (a) FRET donor nucleoside 1; (b) FRET acceptor PyC; (c) Spectral overlap of the FRET pair with the absorbance (dashed) and emission (solid) of nucleoside 1 (red) and PyC (black).
Figure 4.2 Titration of PyC into a solution containing nucleoside 1, with concentrations of PyC ranging from 0 μM (black) to 253 μM (olive).

4.5 – Oligonucleotide design and synthesis

After selecting nucleosides to serve as a FRET pair, a double stranded RNA construct was designed to serve as a substrate for DED1 helicase. Previous studies monitoring DED1 helicase activity through radio-labeling, have established that DED1 effectively loads onto unpaired 3’ overhang regions of RNA strands that are rich in adenine residues.\textsuperscript{23} In order to construct the shortest stable duplex with an effective overhang region, oligonucleotides 2 and 3 were designed (figure 4.3). As PyC is commercially available, it was placed into the longer 35mer oligonucleotide 3. Nucleoside 1 was converted into a protected phosphoramidite using standard chemistry (scheme 4.1) to allow for incorporation into oligonucleotide 2 via solid-phase RNA synthesis. Previously synthesized heterocycle 4\textsuperscript{4} was glycosylated to provide nucleoside 1 after ester saponification. The 5’-OH was protected under
standard conditions with 4,4'-dimethoxytrityl chloride (DMTrCl) and triethylamine in pyridine. The 2'-OH was protected as the (triisopropylsiloxy)methyl (TOM) derivative. Finally, the 3'-OH was activated to yield phosphoramidite 7. After standard solid-phase RNA synthesis, the resulting modified oligonucleotides were purified by PAGE. The full length and proper incorporation of the modified nucleosides were confirmed by MALDI-TOF mass spectrometry.

2 3' - AAU* GCC ACG A-5'  
3 5' - UUA CGG UGC* UUA AAA CAA AAC AAA ACA AAA CAA AA-3'  

**Figure 4.3** Sequences of modified oligonucleotides 2 (10mer) and 3 (35mer), where U* represents modified uridine nucleoside 1 and C* represents PyC.

**Scheme 4.1** Synthesis of phosphoramidite 7. Reagents: (a) (i) N,O-bis(trimethylsilyl)acetamide, CF$_3$SO$_3$Si(CH$_3$)$_3$, β-D-ribofuranose 1-acetate 2,3,5-tribenzoate, CH$_3$CN; (ii) conc. NH$_4$OH, 70%. (b) DMTrCl, Et$_3$N, pyridine, 83%. (d) iPr$_2$NET, nBu$_2$SnCl$_2$, (iPr$_3$SiO)CH$_2$Cl, ClCH$_2$CH$_2$Cl, 30%. (e) iPr$_2$NET, (iPr$_2$N)P(Cl)(CH$_2$CH$_2$CN, ClCH$_2$CH$_2$Cl, 58%.
4.6 – Initial photophysics and duplex stabilities

After synthesis, deprotection, purification, and characterization of modified oligonucleotides 2 and 3, the ability of the two strands to form a duplex was probed. The resulting forward and reverse thermal denaturation curves indicated proper duplex formation and stability (figure 4.4a). To monitor duplex melting through FRET, duplex 2•3 was pre-formed and placed in the fluorimeter cell block at 22 °C. The sample was then heated gradually to 55 °C, monitoring fluorescence every 3 °C (figure 4.4b,c). Upon excitation at 297 nm at 22 °C, the duplex displayed a shoulder near 450 nm most likely resulting from FRET between the two fluorescent nucleoside analogs (nucleoside 1 and PyC). As the duplex was heated and separated into two strands, the FRET signal between the fluorescent nucleosides on the two opposite strands was lost. Although there was a clear change in fluorescence as the duplex melted, it is important to note that the overall fluorescence signal was weak and there was only a small decrease in fluorescence intensity at 445 nm.
Figure 4.4 a) Thermal denaturation curve of duplex 2-3 forward (blue) and reverse (orange) in MOPS buffer (10mM MOPS, 1mM EDTA, 50mM KCl, pH 6.5); b) Normalized fluorescence emission spectra of duplex 2-3 heated from 22 °C (blue) to 55 °C (purple), excited at 297 nm in Tris Buffer (40 mM Tris-HCl, 50 μM MgCl₂, 25mM NaCl, pH 8.0); c) normalized fluorescence intensity at 355 nm (blue dot) and at 455 nm (black dot) as the duplex is being heated.

4.7 – Attempts to visualize FRET signal in the presence of DED1

Starting with cDNA from *S. cerevesiae*, the DED1 gene with a 6-His tag was amplified via PCR. Using this construct, DED1 was expressed in *E. coli* and purified
using established methods.\textsuperscript{25} The ATPase activity of the purified protein was monitored through a continuous spectrophotometric assay, in which pyruvate kinase and lactate dehydrogenase link ATP hydrolysis to oxidation of NADH.\textsuperscript{26} Next, the fluorescence of the modified RNA duplex 2\textsuperscript{-}3 was monitored in the presence of DED1 without ATP and over time upon addition of ATP (figure 4.5). Although the emission spectra exhibited a transformation over time, the change in fluorescence at 445 nm was virtually impossible to differentiate. The strongest emission present appeared to be from the DED1 helicase itself, as it contains seven tryptophan residues. Tryptophan is a fluorescent amino acid with absorption maximum near 280 nm and a quantum yield of 0.12.\textsuperscript{27} Most importantly, the fluorescence emission of tryptophan is sensitive to the microenvironment. Crystal structures of DEAD-box helicases analogous to DED1 indicate that there is a tryptophan residue near the ATP binding site.\textsuperscript{28} Additionally, DEAD-box helicases undergo a conformational change upon ATP binding. The most prevalent change in fluorescence signal appears to correlate with ATP binding rather than duplex melting (figure 4.5b). Although the reaction mixture was excited at 297 nm (nearly 20 nm away from the absorption maximum of tryptophan), the tryptophan emission overwhelmed the emission of the fluorescent oligonucleotide construct. Attempts to excite the reaction mixture at longer wavelengths resulted in loss of any clear FRET signal.
Figure 4.5 a) Fluorescence emission spectra of 2-3 alone (purple), 2-3 with DED1 (black) and after the addition of ATP (blue) from 15 seconds to 7 minutes in (40 mM Tris-HCl, 50 μM MgCl₂, 25 mM NaCl, pH 8.0) excited at 297 nm at 25 °C; b) simplified emission spectra showing the change in fluorescence of DED1 (black) upon ATP binding (blue).

4.8 – Implications and possible system re-design

Clearly, an alternate FRET pair must be designed in order to effectively measure DED1 helicase activity at the nucleotide level. Two factors must be taken into consideration upon design of a new FRET pair. First, the acceptor should have an excitation wavelength far above 300 nm to avoid exciting tryptophan residues in the helicase. Second, the donor fluorescent nucleoside analog should have a significantly higher quantum yield, while the acceptor should have a higher molar absorptivity. This would help to increase the overall fluorescence signal of the RNA construct, dwarfing the emission of the native tryptophan residues in the helicase.

For example, another quinazoline-based fluorescent nucleoside with the methoxy group shifted to a different position on the heterocycle (8) has a fluorescence quantum yield twice that of nucleoside 1 (figure 4.6a). Nucleoside 8 has a red-shifted
absorption maximum in water near 320 nm and could serve as an effective FRET donor with an emission maximum at 395 nm. Additionally, nucleoside 9, an extended PyC analog has a large extinction coefficient of $12.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 371 nm (figure 4.6b) and a minimum in optical density near 320 nm, allowing nucleoside 9 to serve as a potential FRET acceptor. Nucleoside 9 has an emission maximum near 473 nm, which could provide a more distinct FRET signal than that observed from construct 2•3.

![Chemical structures](image)

Figure 4.6 a) Nucleoside 8 and its photophysical properties in water; b) Nucleoside 9 and its photophysical properties in water.

It is important to note, that as the quantum yield and excitation wavelength increase, the isomorphicity of the system decreases. In this case, adding an additional thiophene moiety to PyC dramatically increases the extinction coefficient. There is a balance between isomorphicity and photophysical properties that must be determined for every assay. Fortunately, control helicase activity assays can be run with radio-labeled constructs to compare the rate of the fluorescently modified RNA to that of the native constructs.
4.9 – Concluding remarks

In moving towards the compatibility of fluorescent nucleosides with native proteins, there are a number of significant challenges. Ideally, fluorescent nucleoside analogs must have a high quantum yield and an excitation wavelength significantly greater than 300 nm. If a fluorescent nucleoside analog is to be used alone in an assay, that nucleoside analog must display sensitivity towards the microenvironment. However, for fluorescent nucleoside analogs to be implemented in a FRET system, it is of utmost importance to choose a nucleoside analog that displays minimal sensitivity to the microenvironment.

If a fluorescent nucleoside analog FRET pair can be successfully used to monitor the RNA helicase activity of DED1, there is great potential to expand this assay to be used for any number of potential RNA helicases. Currently, many proteins have been designated as RNA helicases only because of amino acid sequence homology. However, it remains to be seen if these enzymes actually behave as RNA helicases. It would be of great benefit to have a simple, accessible assay to probe the ability of a helicase to actually locally melt RNA duplexes.

In addition to using two fluorescent nucleoside analogs as a FRET pair, there is great potential in using a fluorescent nucleoside analog as a FRET donor with a common tag on a protein. The activity of proteins known to interact with nucleic acids, such as DNA repair enzymes, transcription factors, and ribonucleases, could be monitored with FRET accuracy in real-time. As the toolbox of fluorescent
nucleoside analogs continues to grow, such assays become more realistic to implement.

Appendix – Experimental information

A.1 – General procedures

Reagents were purchased from Sigma-Aldrich, Rasayan Inc. (Encinitas, CA), Acros, and VWR and were used without further purification unless otherwise specified. Solvents were purchased from Sigma-Aldrich and Fisher Scientific. Anhydrous acetonitrile was obtained from Glen Research (Sterling, VA). Anhydrous N,N-dimethylformamide was obtained using a two-column purification system (Glasscontour System, Irvine, CA). NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Reactions were monitored with analytical thin-layer chromatography (TLC) performed on pre-coated silica gel aluminum-backed plates (Merck Kieselgel 60 F254). All experiments involving air and/or moisture sensitive compounds were carried out under an argon atmosphere. Column chromatography was performed with silica gel particle size 40-63 μm. NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Mass spectra were obtained on an Agilent 6230 HR-ESI-TOF MS at the Molecular Mass Spectrometry Facility at the UCSD Chemistry and Biochemistry Department. All absorption measurements were obtained at 21 °C on a Shimadzu UV 2450 absorption spectrometer using a quartz cuvette with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany). Steady state fluorescence measurements were obtained at 21 °C on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer using a
quartz fluorescence cell with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany). All CD spectra were recorded on an Aviv 215 Circular Dichroism Spectrometer in a 350 µL quartz cell with a path length of 0.1 cm at 25 °C.

A.2 – Synthetic procedures

**7-Methoxyquinazoline-2,4(1H,3H)-dione ribonucleoside (1).** To a suspension of 4 (0.20 g, 1.0 mmol) in anhydrous acetonitrile (15 mL), N,O-Bis(trimethylsilyl)acetamide (1.3 mL, 5.0 mmol) was added dropwise under argon. The reaction was stirred at 25 °C for 30 min. TMSOTf (0.12 mL, 0.10 mmol) and β-D-ribofuranose-1-acetate-2,3,5-tribenzoate (0.50 g, 1.0 mmol) were added at the same time under argon. The reaction temperature was raised to 50 °C. The reaction was stirred at 50 °C for 5 h. TMSOTf (0.12 mL, 0.10 mmol) and β-D-ribofuranose-1-acetate-2,3,5-tribenzoate (0.50 g, 1.0 mmol) were added at the same time under argon again. The reaction was stirred at 50 °C for 28 h. The reaction was cooled to room temperature, concentrated to an oil, and diluted with dichloromethane (20 mL). The solution was washed with saturated sodium bicarbonate and brine. The organic layer was dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was run through a silica plug (50% ethyl acetate in hexanes). The solvent was removed under reduced pressure, and the crude product was dissolved in dioxane (15 mL) and transferred to a 200 mL pressure tube. Ammonium hydroxide (28%, 55 mL) was added to the tube. The reaction was stirred at 70 °C for 24 h. The solvent was removed under reduced pressure, and the product was isolated by flash chromatography (5-12% methanol in dichloromethane).
Product: white solid (0.23 g, 0.70 mmol, 70 % yield over two steps). $^1$H-NMR (400 MHz, DMSO-$d_6$): δ 11.52 (s, NH, 1H), 7.91 (d, $J = 8.8$ Hz, 1H), 7.07 (s, 1H), 6.87 (dd, $J_1 = 8.0$ Hz, $J_2 = 0.8$ Hz, 1H), 6.17 (d, $J = 6.4$ Hz, 1H), 5.25 (d, $J = 4.4$ Hz, 1H), 5.03 (d, $J = 4.8$ Hz, 1H), 4.99 (t, $J = 4.8$ Hz, 1H), 4.46 (d, $J = 3.6$ Hz, 1H), 4.14 (s, 1H), 3.84 (s, 3H), 3.70 (dd, $J_1 = 5.6$ Hz, $J_2 = 4.4$ Hz, 1H), 3.59 (m, 1H); $^{13}$C-NMR (100 MHz, DMSO-$d_6$): δ 164.93, 161.79, 151.44, 142.30, 130.06, 111.84, 110.24, 101.38, 90.24, 84.75, 69.10, 68.84, 61.10, 56.68; ESI-MS calculated for C$_{14}$H$_{16}$N$_2$O$_7$ [M+H]$^+$ 324.10 and [M+Na]$^+$ 347.09, found 324.87 and 347.03, respectively.

5′-Dimethoxytrityl-7-methoxyquinazoline-2,4(1H,3H)-dione ribonucleoside (5). Anhydrous pyridine (3 mL), anhydrous triethylamine (51 μL, 0.37 mmol) and 4,4′-dimethoxytrityl chloride (0.12 g, 37 mmol) were added to 1 (0.10 g, 0.31 mmol) under argon. The reaction was stirred at room temperature overnight and quenched with methanol (0.5 mL). The solvent was removed under reduced pressure, and the product was isolated by flash chromatography (1% triethylamine, 2% methanol, 97 % dichloromethane). Product: white solid (0.16 g, 0.26 mmol, 83 % yield). $^1$H-NMR (400 MHz, CDCl$_3$): δ 7.46 − 7.16 (m, 12H), 6.97 (t, $J = 8.8$ Hz, 1H), 6.75 (d, $J = 8.8$ Hz, 2H), 6.60 (d, $J = 8.4$Hz, 1H), 6.32 (d, $J = 5.6$ Hz, 1H), 4.81 (t, $J = 6.4$ Hz, 1H), 4.57 (t, $J = 6.4$ Hz, 1H), 4.05 (br, 1H), 3.88 (s, 3H), 3.73 (s, 6H), 3.47−3.55 (m, 2H); $^{13}$C-NMR (100 MHz, CDCl$_3$): δ 161.48, 160.50, 158.66, 150.60, 144.99, 142.68, 136.04, 135.97, 135.81, 130.48, 128.56, 128.02, 127.01, 113.30, 109.53, 106.56, 90.79, 86.48, 83.56, 69.96, 69.39, 63.42, 63.29, 56.58, 55.45, 53.13; ESI-MS calculated for C$_{35}$H$_{34}$N$_2$NaO$_9$ [M+Na]$^+$ 649.2, found 649.2.

2′-(Trisisopropysiloxy)methyl-5′-dimethoxytrityl-7-methoxyquinazoline-2,4(1H,3H)-dione ribonucleoside (6). Anhydrous dichloroethane (3 mL) and N,N-
diisopropylethylamine (0.17 mL, 1.0 mmol) were added to 5 (0.20 g, 0.32 mmol) with stirring under argon. Dibutyltin dichloride (0.10 g, 0.33 mmol) was added to the reaction via syringe and stirred at room temperature for 1 h. The reaction was placed into a 80 °C water bath and stirred for 10 min. (Triisopropylsiloxy)methyl chloride (87 μL, 38 mmol) was added, and the reaction was stirred at 80 °C for 15 min. The reaction was diluted with dichloromethane (10 mL) and poured into saturated sodium bicarbonate (15 mL). The mixture was stirred vigorously for 15 min. The organic layer was extracted, and the aqueous layer was washed with dichloromethane (5 mL). The organic layers were pooled and dried over sodium sulfate. The solvent was removed under reduced pressure, and the product was isolated by flash chromatography (1% triethylamine, 35% ethyl acetate, 64 % hexanes). Product: white foam (0.078 g, 0.01 mmol, 30 % yield). ¹H-NMR (400 MHz, CDCl₃): δ 7.43 – 7.47 (m, 3H), 7.26 – 7.34 (m, 4H), 7.18 – 7.26 (m, 4H), 7.06 (t, J = 8.4 Hz, 1H), 6.78 (d, J = 8.8 Hz, 3H), 6.67 (d, J = 8.4 Hz, 1H), 6.35 (d, J = 5.2 Hz, 1H), 5.07 (d, J = 4.8 Hz, 1H), 4.90 – 4.95 (m, 2H), 4.61 (t, J = 6.4 Hz, 1H), 4.07 (dd, J₁ = 4.0 Hz, J₂ = 3.2 Hz, 1H), 3.95 (s, 3H), 3.77 (s, 6H), 3.51 (dd, J₁ = 8.0 Hz, J₂ = 2.4 Hz, 1H), 3.39 (dd, J₁ = 6.4 Hz, J₂ = 4.0 Hz, 1H), 3.28 (q, J = 7.2 Hz, 1H), 2.70 (q, J = 6.8 Hz, 1H), 1.07 – 1.00 (m, 21H); ¹³C-NMR (100 MHz, CDCl₃): δ 161.71, 160.02, 158.72, 149.92, 144.96, 143.91, 136.00, 135.94, 130.45, 128.53, 128.05, 127.08, 113.33, 109.71, 106.75, 106.13, 91.08, 88.99, 86.63, 83.70, 69.70, 63.44, 59.85, 56.71, 55.45, 29.95, 17.95, 12.02; ESI-MS calculated for C₄₅H₅₆N₂O₁₀Si [M+Na]⁺ 835.4, found 835.4.

3′-2-Cyanoethylidiisopropylphosphoramidite-2′-(Triisopropylsiloxy)methyl-5′-dimethoxytrityl-7-methoxyquinazoline-2,4(1H,3H)-dione ribonucleoside (7).

Anhydrous dichloromethane (0.6 mL) and N,N-diisopropylethylamine (0.13 mL, 0.75
mmol) were added to 6 (0.05 g, 0.062 mmol). The reaction was cooled on ice, and 2-cyanoethyl \(N,N\)-diisopropylchlorophosphoramidite (28 \(\mu\)L, 0.13 mmol) was added. The reaction was stirred at room temperature for 15 h. The solvent was removed under reduced pressure, and the product was isolated by flash chromatography (1% triethylamine, 15–30% ethyl acetate in hexanes). Product: white foam (0.038 g, 0.037 mmol, 58% yield). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.44–7.46 (m, 3H), 7.32–7.34 (m, 4H), 7.22–7.26 (m, 4H), 7.07 (t, \(J = 8.4\) Hz, 1H), 6.78 (d, \(J = 8.1\) Hz, 3H), 6.68 (d, \(J = 8.4\) Hz, 1H), 6.35 (d, \(J = 5.4\) Hz, 1H), 5.07 (d, \(J = 4.2\) Hz, 1H), 4.91–4.94 (m, 2H), 4.62 (t, \(J = 6.4\) Hz, 1H), 4.07 (b, 1H), 3.96 (s, 3H), 3.78 (s, 6H), 3.51 (m, 1H), 3.40 (m, 1H), 2.81 (q, \(J = 6.9\) Hz, 1H), 2.01 (b, 1H), 1.20–1.15 (m, 8H), 1.01 (d, \(J = 6.8\) Hz, 4H), 0.92–0.89 (m, 21H); \(^{13}\)C-NMR (100 MHz, CDCl\(_3\)): \(\delta\) 171.37, 161.62, 160.19, 158.67, 158.65, 144.88, 136.10, 136.03, 135.88, 135.50, 130.47, 130.44, 128.67, 128.56, 127.99, 127.94, 127.05, 126.99, 117.94, 117.53, 113.23, 106.61, 106.23, 86.50, 86.46, 64.56, 60.60, 59.02, 56.66, 55.40, 55.35, 43.58, 43.45, 43.31, 43.19, 30.84, 29.90, 24.82, 24.75, 24.69, 21.24, 21.21, 19.23, 17.87, 17.81, 14.40, 13.92, 12.06, 12.01, 11.98; ESI-MS calculated for \(C_{54}H_{73}N_4O_{11}PSi\)[M+Na]\(^+\) 1035.5 and [M+K]\(^+\) 1051.4, found 1035.4 and 1051.4.

### A.3 – Oligonucleotide synthesis and purification

The modified oligonucleotides were synthesized on an Expedite 8909 DNA synthesizer using a 0.2 \(\mu\)mole scale 500 Å CPG column. Phosphoramidite 7 was site specifically incorporated into the oligonucleotide by trityl-off synthesis of the base oligonucleotide, followed by manual coupling of phosphoramidite 7. Typically, the
modified phosphoramidite was dissolved in 100 μL of anhydrous acetonitrile to give a final concentration of 0.1M. The phosphoramidite solution was pushed into the CPG column via syringe and then 200 μL of 0.45M 1H-tetrazole was pushed into the other end of the column via syringe. Coupling reactions, performed twice, were allowed to proceed for 5 minutes (97% coupling efficiency) and were subsequently followed by standard oxidation and capping steps. The rest of the oligonucleotide was synthesized via the standard trityl-off procedure.

Upon completion of the oligonucleotide synthesis, the CPG column was dried completely. The beads from the column were transferred into a 2.5 mL conical glass vial. 1mL of MeNH₂ in water and 1mL of MeNH₂ in ethanol were added. The vial was capped tightly and allowed to react for 15 h at room temperature. The supernatant from the reaction vial was transferred into Eppendorf tubes. The remaining beads were washed with 3x500 μL of 33% ethanol in water. The supernatant was evaporated in speed vac. The solid residue was dissolved in 1 mL of 1M TBAF in THF and warmed to 50 °C for 5min. The deprotection took place overnight at 25 °C. The reaction was quenched by adding 1 mL of Tris buffer (1M, pH 7.4). The volume was reduced to half by speed vac. The residue was desalted on a G10 sephadex column. The residue was loaded onto the column in 1 mL of water and eluted with 10 mL of water. The fractions were collected and analyzed by UV (260 nm). Fractions containing RNA were evaporated and desalted again on a G10 sephadex column. The desalted RNA was pooled together and evaporated in speed vac. The RNA was purified by 20% polyacrylamide gel electrophoresis. The oligonucleotide was visualized by UV shadowing; bands were excised from the gel and extracted with 0.3M sodium acetate buffer overnight. The resulting solution was filtered (Bio Rad
poly-prep chromatography column) and desalted using a Sep-Pak cartridge (Waters Corporation, MA). The following 260 nm extinction coefficients were used to determine the concentration of oligonucleotides: \( r_G = 11,700 \), \( r_C = 7,300 \), \( r_A = 15,400 \), \( r_U = 10,100 \), and \( 1 = 10,700 \).

**A.4 – Oligonucleotide hybridizations**

Oligonucleotide samples were prepared in 25 \( \mu \)M solutions in MOPS buffer (10mM MOPS, 1mM EDTA, 50mM KCl, pH 6.5). Samples were heated to 90 °C for 3 minutes and cooled to room temperature over 2–3 hrs. Samples were then placed at 0 °C until spectroscopic measurements were obtained. DED1 assays were performed by adding the annealed RNA (5x) directly to 5 \( \mu \)M solutions of DED1 in Tris buffer (40 mM Tris-HCl, 50 \( \mu \)M MgCl\(_2\), 25mM NaCl, pH 8.0). To this solution a freshly prepared 10X ATP solution (50mM ATP, 50mM MgCl\(_2\)) was added. Steady-state fluorescence spectra were obtained on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer using a 125 \( \mu \)L quartz fluorescence cell with a 1.0 cm path length (Hellma GmbH & Co KG, Müllheim, Germany) at 25 °C with slit widths of 12 nm. Thermal denaturation curves were obtained on a Beckman-Coulter DU® 640 spectrometer with a high performance temperature controller and a micro auto six sample holder. Samples were heated from room temperature to 80 °C at a rate of 0.5 °C min\(^{-1}\) with optical monitoring every minute at 260 nm.
4.10 – References


Chapter 4 contains sections of text that are reprinted from: Noé, M. S.; Xie, Y.; Tor, Y. In *Methods for Studying Nucleic Acid/Drug Interactions*; Wanunu, M.; Tor, Y. Ed.; CRC Press (Taylor & Francis Group): Boca Raton, FL, 2012. The dissertation author is the main author and researcher for this work.