Progress Toward Synthesis of Molecular Beacons Incorporating DABCYL Analog Quenchers

(Thesis format: Monograph)

by

Christie Ettles

Graduate Program in Chemistry

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Progress toward the synthesis of molecular beacons containing analogs of the universal quencher 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) has been made. A synthetic methodology has been developed for the differential labeling of a PNA molecular beacon with quencher and fluorophore moieties. A DABCYL analog diarylazo compound and three small fluorescent molecules have been derivatized with terminal alkyne groups for incorporation into azide-decorated PNA oligomers by on-resin “click” chemistry. An optimized on-resin procedure was used to determine the viability of the fluorophores and quencher for incorporation into PNA.

The synthesis of a pyrrolocytidine phosphoramidite containing a diarylazo DABCYL analog moiety was undertaken with the aim of incorporation into a DNA molecular beacon by standard phosphoramidite chemistry. While the phosphoramidite was not achieved, an unprecedented addition of the dimethoxytrityl protecting group to the electron-rich pyrrolocytidine heterocycle was observed and a mechanism for its formation is proposed.

Keywords

Nucleic acid, peptide nucleic acid, CuAAC, fluorescence, quencher, nucleoside analog, molecular beacons, DABCYL
Acknowledgments

First and foremost I have to thank Dr. Hudson, for accepting me into his research group and for his help, encouragement, and patience over the past two years. Under his tutelage I have learned more about chemistry than I even thought possible. Also thanks to my family and friends back home in New Brunswick for cheering on my scientific ventures here in Upper Canada.

Here at Western, Dr. Mojmir Suchy has also been a great source of advice and helpful discussion. McKenry Charles and André St. Amant were excellent mentors during my first months of grad school. I must give love to my current and former Lab 202 inmates Melissa and Kirby. Rachael, Augusto, Adam, and Dr. Mark have been amazing labmates as well. Thanks to Mat Willans for helping with NMR spectra and Kristina in the Yeung group for emergency MALDI. I must also thank Dr. Felix Lee, Rob Harbottle, and Lesley Tchorek for offering advice and good cheer when it was most needed.

Finally, thanks to Daphne and Meredith for being so cute and fuzzy.
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List of Abbreviations

A – adenine

ABI – Applied Biosystems International

br s – broad singlet

Bz – benzoyl

C – cytosine

CFTR - cystic fibrosis transmembrane conductance regulator

CLEAR - cross-linked ethoxylate acrylate resin

CuAAC – copper-catalyzed azide-alkyne cycloaddition

d – doublet

DABCYL – 4-(4’-dimethylaminophenylazo)benzoic acid

DCM – dichloromethane

dd – doublet of doublets

DIPEA – diisopropylethylamine

DMF – dimethylformamide

DMSO – dimethylsulfoxide

DMT – dimethoxytrityl

DNA – deoxyribonucleic acid

EAS – electrophilic aromatic substitution

EDANS – aminonaphthalene-1-sulfonic acid
EDTA – ethylenediaminetetraacetic acid

F - fluorophore

FCC – flash column chromatography

FRET – Förster resonance energy transfer

IC – internal conversion

ISC – intersystem crossing

LC-MS – liquid chromatography mass spectrometry

m – multiplet

MALDI – matrix-assisted laser desorption/ionization

MB – molecular beacon

MeCN – acetonitrile

MeOH – methanol

mRNA – messenger RNA

MS – mass spectrometry

NBD - 4-nitrobenzo-2-oxa-1,3-diazole

nm – nanometers

NMR – nuclear magnetic resonance

pC – pyrrolocytidine

Q – quencher

PNA – peptide nucleic acid
RBF – round-bottomed flask

RNA – ribonucleic acid

RT – room temperature

s – singlet

SNP – single nucleotide polymorphism

T – thymine

t – triplet

TBDMS – tert-butyl dimethylsilane

TCA – trichloroacetic acid

TEA – triethylamine

TES – triethylsilane

TFA – trifluoroacetic acid

THF – tetrahydrofuran

TLC – thin layer chromatography

TMS – trimethylsilyl

UPLC – ultra performance liquid chromatography

UV – ultraviolet

µmol – micromole
Chapter 1 - Introduction

1.1 Nucleic Acids

Nucleic acids are the biomacromolecules that serve as template and repository for the genetic material of all living beings, from bacteria to plants to humans. Deoxyribonucleic acid (DNA) is the nucleic acid component of chromosomes, and codes for genes; ribonucleic acid (RNA), has myriad functions but is primarily known as the biopolymer that makes up the genetic transcripts that are translated into proteins.¹

![Figure 1. The basic structures of deoxyribonucleic and ribonucleic acid nucleotides.](image)

The concept of “genes” as carriers of hereditary traits was first explored by Gregor Mendel in 1865, but at that time the biochemical basis of genetic material and its reproduction was far from being understood.² The first major development in the discovery of nucleic acids came in 1869. Johann Friedrich Miescher, a German physician working in the relatively new realm of “physiological chemistry”, had embarked on a project to elucidate the biochemical composition of cells by isolating leukocytes from pus in surgical bandages. In his attempts to isolate lipids and proteins from these cells, he encountered a substance that appeared to precipitate in acidic solution, then redissolve when basified. Elemental analysis of this substance identified it as distinct from both proteins and lipids in that it contained a high percentage of phosphorus. Miescher dubbed this substance “nuclein”, as he had isolated it from the nuclei of cells. However, Miescher’s published theories as to the purpose of “nuclein” touched only lightly on the possibility of its involvement in gene expression or cellular reproduction.³ In 1889, Richard Altmann was able to isolate a sample of this material untainted by proteins.⁴ Unaware that he had obtained a pure form of Miescher’s “nuclein”, ³ he recognized the acidic character of the substance and dubbed it Nucleinsäuren – nucleic acid.
Later advancements in the nascent field of nucleic acid chemistry included the work of Albrecht Kossel, who identified the four compounds now known as the canonical DNA nucleobases: the purines, adenine and guanine, and the pyrimidines, cytosine and thymine.\(^5\)

![Diagram of DNA nucleobases: adenine, guanine, cytosine, and thymine.]

**Figure 2.** The DNA nucleobases: adenine, guanine, cytosine, and thymine.

Phoebus A. Levine further elucidated the components of “yeast nucleic acid”, as ribonucleic acid (RNA) was then known.\(^5\) Via base hydrolysis of RNA he was able to identify the phosphate and sugar components. Levine also correctly described their connectivity, although he incorrectly asserted that nucleic acids formed in “tetranucleotides” – cyclic structures consisting of one of each of the four nucleotides joined covalently.\(^6\) Finally, Erwin Chargaff was the first to recognize that in any given sample of DNA, the ratio of A:T and C:G bases remained constant.\(^7\)

The elucidation of the structure of DNA is a relatively recent but profoundly important development in biochemistry. By the mid-twentieth century, the chromosomal nature of genes had been established but the scientific community was divided on whether the genetic material was carried by the nucleic acids or proteins found within chromosomes. A definitive structure for nucleic acids might provide an explanation for the problem of which macromolecule carried genes, and how said genes were replicated.

James Watson and Francis Crick, at the University of Cambridge, applied themselves to the DNA structure problem in the early 1950s. It was not until Maurice Wilkins, a researcher at Kings College in London, showed them a photograph Wilkins’s colleague Rosalind Franklin had obtained of DNA that they were able to deduce the helical structure of the molecule.\(^8\)

Watson and Crick also felt that the phosphates in DNA were found on the outside of the molecule. However, what eluded them at the time was an understanding of what held the two
strands together. Watson proposed that the nucleobases paired with themselves in the helix (adenine with adenine, for example).\(^8\)

Watson and Crick consulted Jerry Donohue, a visiting chemist from Pauling’s lab, to determine the most stable tautomers of the nucleobases. On learning that the keto forms were most stable, and realizing that in these forms the bases could hydrogen bond with each other in A-T and G-C purine-pyrimidine pairs, Watson and Crick were able to put the finishing touches on their model.\(^8\)

Watson and Crick published their proposed structure in the issue of *Nature* dated April 25, 1953 – a single-page paper that modestly concludes: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a copying mechanism for the genetic material.”\(^9\)

**Figure 3.** Watson and Crick's proposed structure for the DNA double helix, with detail showing the two antiparallel strands joined by hydrogen bonding between base pairs (G-C and A-T depicted).
Watson and Crick’s paper paved the way for further studies that established the supreme importance of nucleic acids in biological processes. Crick himself eventually formulated a simple scheme that he described as the “central dogma of molecular biology”:  

![Diagram of DNA Transcription to mRNA Translation to Protein]

**Figure 4.** The central dogma of molecular biology.

Ultimately, nucleic acids are organic molecules, and as such can be synthesized, modified, and manipulated – a rich field of study that has been expanding ever since Francis Crick announced in the Eagle pub that he and his partner James Watson had “discovered the secret of life.”

### 1.2 Hybridization Probes

The extraordinary specificity and stability of hydrogen bonding interactions between purine and pyrimidine base pairs in nucleic acids, first elucidated by Watson and Crick, is a much-studied facet of nucleic acid hybridization. The potential of this molecular recognition event in the design of probes to identify specific nucleotide sequences was realized as early as 1963, when radiolabeled RNA oligonucleotides were reported as a method to identify complementary sequences of DNA in samples. Hybridization probes, as they are now known, would see a surge in interest and development over the next two decades, aided by such discoveries as endonuclease digestion of DNA. In 1975, Edward Southern’s report of a hybridization method to affix radiolabeled probes directly to digested DNA, known as a Southern blot, was among the most significant advances in this field.

However, hybridization probe technology initially suffered from two major drawbacks. First, available methods for labeling probes – mostly involving radioactivity or fluorescence – did not provide a method for “turning off” the probes, requiring the removal of unhybridized probe from samples to prevent background signals. Second, this requirement also called for the hybridized nucleic acids to be immobilized on a membrane or other surface. These requirements precluded the use of hybridization probes to detect nucleic acid targets in real time, or in living cells.
1.3 Molecular Beacons

A promising advance in the field of hybridization probes came in 1996, when Tyagi and Kramer published a report of a novel hybridization probe called a “molecular beacon.” The design they proposed was simple: a DNA oligonucleotide, with a “probe sequence” of 15-35 nucleotides and “stem sequences”, 5-10 nucleotides in length, whose complementary sequences enabled the oligonucleotide to anneal in a hairpin structure. The oligonucleotide also contained a fluorophore, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), attached by a hydrocarbon linker to its 5' phosphate group; a quencher molecule, 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), was attached by a similar linker to the 3' hydroxy group of the probe.

Figure 5. Tyagi and Kramer’s proposed molecular beacon structure.

When in the hairpin conformation, the fluorophore and quencher were brought into adequate proximity to enable quenching of the EDANS fluorescence by the DABCYL moiety via Förster resonance energy transfer (FRET). However, in the presence of DNA containing a sequence complementary to the probe sequence, hybridization of the probe to the target would cause the
hairpin to unwind, increasing the distance between the fluorophore and quencher. Restoration of fluorescence would thus signal the successful binding of the oligonucleotide to its target.

This new technology offered two major benefits. First, the fact that the molecular beacon was “switched off” in its native conformation removed the necessity to wash away unhybridized probe. Second, the added feature of the hairpin stem enabled fine-tuning of the probe-target hybridization stability so that the probe could be designed to recognize target mismatches as minute as a single base pair. As such, molecular beacons could conceivably have applications in both real time and \textit{in vivo} assays. They could also be synthesized with relative ease, using established automated DNA synthetic techniques, and purified via column chromatography.

Since their introduction in 1996, interest in molecular beacons has grown rapidly. Among other applications, MBs have been described as probes for single nucleotide polymorphisms (SNPs), \footnote{mRNA detection in living cells,} and identification of viruses in cells. \footnote{16}

1.4 Fluorescence and Quenching

1.4.1 Fluorescence

Fluorescence is a form of luminescence in which a molecule absorbs a photon at a particular wavelength, passes to an excited state, and emits the photon at a longer wavelength. \footnote{To understand the difference between fluorescence and other forms of energy dissipation, a closer examination of processes by which an excited light-absorbing molecule (chromophore) can return to the ground state is merited.}

A chromophore at the ground state ($S_0$), on absorbing a photon, will be transformed to an excited state ($S_2$). The molecule will then return to the lowest possible excited state ($S_1$) by dissipating energy as heat in a phenomenon known as internal conversion (IC).
Figure 6. Jablonski diagram showing electronic transitions in the absorption and emission of photons by chromophores.

Once \( S_1 \) is reached, the molecule can return to the ground state by various modes of energy dissipation. Three of the most common are:

1. The remaining absorbed energy is dissipated as heat in a non-radiative process.

2. The photon undergoes intersystem crossing (ISC) to an excited triplet state \( (T_1) \), and the energy is emitted as phosphorescence.

3. The photon returns from \( S_1 \) to \( S_0 \) by a radiative process, as fluorescence.

The longer wavelength at which fluorescence is emitted can be explained by the loss of energy as heat during IC. Chromophores that emit energy as fluorescence are known as fluorophores.

Certain photophysical properties will be constant for fluorophores under a given set of conditions, and can be measured. Changes in these properties can signal changes in the immediate environment of the fluorophore, and thus fluorescence can be extraordinarily useful in bioanalysis. Among the most readily characterized fluorescence properties are absorption and emission maxima/Stokes shift; fluorescence quantum yield; and molar extinction coefficient.

The absorption maximum \( (\lambda_{\text{max}}) \) of a fluorophore indicates the wavelength of light at which it can most efficiently be excited. The emission maximum indicates the wavelength at which light
will be emitted from $S_1$. The difference in these two wavelengths is called the Stokes shift. Changes in this shift can be used in such applications as detecting cancerous tissues.

Fluorescence quantum yield ($\Phi$) is a measurement of the efficiency of a fluorescence process. It can be quantified as the ratio of the number of photons emitted by a fluorescent compound as fluorescence, to the number of photons absorbed by the compound:

$$\Phi = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{k_r}{k_r + k_i + k_{isc}}$$

Where $k_r$, $k_i$, and $k_{isc}$ are the rate constants for fluorescence emission, heat emission, and intersystem crossing respectively. Thus a high $\Phi$ is often indicative of an efficient, and therefore highly fluorescent, fluorophore.

The molar extinction coefficient, or molar absorptivity ($\epsilon$), is a measure of how well a given chromophore absorbs light at a particular wavelength (usually $\lambda_{max}$). It can be related to the Beer-Lambert law, $A = \epsilon c \ell$, where $A$ = absorbance, $c$ = concentration, and $\ell$ = path length (usually 1 cm when measured in a cuvette). A fluorophore with high quantum yield and molar absorptivity is considered to be “bright”, and its fluorescence is expected to be detectable at lower concentrations.

1.4.2 Quenching Modes: FRET and Collisional

Förster resonance energy transfer can be defined as the nonradiative process by which an excited donor molecule transfers electronic excitation to a ground state acceptor molecule. Theodor Förster first described this phenomenon in 1945. He ascribed the transfer to dipole-dipole resonance interaction between the donor and acceptor molecules, and also noted the distance-dependence of the rate of transfer, which he quantified as $1/r^6$, where $r$ is the distance between two molecules. 10-100 Å is considered the maximum range for FRET to occur, while 20-70 Å – the “Förster distance” – is the distance at which FRET occurs with 50% efficiency. This scale was recognized as ideal for measuring distance relationships between biomolecules.

In a molecular beacon, a fluorophore functions as the donor and a quencher acts as the acceptor. Apart from proximity, the major requirement for FRET to occur between the fluorophore and...
A quencher of a molecular beacon is that the emission wavelength of the fluorophore must overlap with the absorption wavelength of the quencher. Energy emitted by the fluorophore on emission will thus be absorbed by the quencher; on relaxation, the quencher will emit the energy as heat, and fluorescence will not be observed.

**Figure 7.** Wavelength overlap and proximity requirements for FRET.

Apart from FRET, another quenching mechanism observed in molecular beacon fluorophore–quencher pairs is collisional quenching, which involves the direct transfer of energy from the fluorophore to the quencher during physical contact of the two molecules. This mode of quenching has been observed between the universal quencher DABCYL and fluorophores whose emission wavelengths do not overlap adequately to enable FRET.

### 1.4.3 DABCYL Quenching Mechanism

_Cis-trans_ photoisomerization around a double bond has been demonstrated as a self-quenching mechanism in fluorophores containing such bonds; this same mechanism can easily be extended to the diarylazo moiety of DABCYL and its analogs.
Azobenzene, considered a model molecule for compound bearing the diarylazo moiety, is known to exist as an equilibrium between the more stable trans isomer and the cis isomer. Upon irradiation with UV light, the trans compound undergoes photoisomerization to the cis isomer. The return process can be mediated either by light or, in the absence of light, by thermal isomerization to the more stable trans form.

Numerous mechanisms have been proposed for this isomerization process, of which two, rotation and inversion, are the most commonly accepted. Rotation involves a breakage of the N=N bond to enable rotation between that bond; inversion proceeds by inversion of a nitrogen lone pair and rotation around the C-N bond. Both are corroborated by experimental evidence, and in some cases a mixture of mechanisms has been proposed.

Scheme 1. Trans-cis isomerization of azobenzene.

Scheme 2. Proposed mechanisms for azobenzene isomerization.
The quenching of fluorescence by DABCYL can thus be attributed to absorption of a photon from the fluorophore which then mediates photoisomerization from the *trans* to *cis* isomer, preventing the photon from being released as fluorescence.

### 1.5 Peptide Nucleic Acid (PNA)

The first molecular beacons consisted of DNA oligonucleotides. DNA molecular beacons are still widely used in such applications as real-time PCR assays and Southern blots, largely due to their wide commercial availability and ease of synthesis. However, while DNA MBs function effectively *in vitro*, they present problems for *in vivo* applications (namely, the tendency of single-stranded DNA to be degraded by nucleases in cells). Numerous modifications have been proposed to the original molecular beacon design; one of the most promising is the use of peptide nucleic acid (PNA) peptides instead of DNA oligonucleotides as molecular beacons.

First reported in 1991, PNA is a nucleic acid analog with a backbone of N-(2-aminoethyl)-glycine units linked by peptide bonds. Purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. The bases can recognize their complements in DNA and RNA via standard Watson-Crick hydrogen bonding; however, the nonstandard backbone is not recognized by nucleases, leading to increased stability of PNA in living cells. Also, the backbone is neutral, preventing the electrostatic repulsion such as that between negatively charged phosphate backbones that can destabilize DNA or RNA duplexes. As such, PNA oligomers can form highly stable duplexes and triplexes with complementary nucleic acid sequences both in water, and in solutions with low salt content.

![Figure 8. Structure of peptide nucleic acid (PNA).](image-url)
A further benefit of PNA-based molecular beacons is that they do not require base-pairing stem sequences for quenching of fluorescence in the absence of their targets. Rather, intramolecular interactions such as base-stacking can cause the oligomer to fold in on itself in solution, bringing the quencher and fluorophore into the close proximity required for FRET.\(^{32}\) This quality can potentially simplify the design of PNA-based molecular beacon sequences.

1.6 Overview

The research described in this report focuses on the synthesis of FRET-based PNA and DNA molecular beacons. Chapter 2 describes a proposed methodology for the synthesis of a PNA MB sequentially derivatized with quencher and fluorophore moieties via on-resin copper-catalyzed azide-alkyne cycloaddition (CuAAC), commonly known as “click chemistry”. Three fluorescent molecules have been derivatized with alkyne moieties for incorporation into azide-containing PNA, and their fluorescence properties characterized. Optimization of the on-resin click chemistry and progress toward the synthesis of differentially labeled PNA molecular beacons are described.

Chapter 3 focuses on the synthesis of a pyrrolocytidine phosphoramidite incorporating the DABCYL diarylazo moiety. Two potential synthetic routes are explored toward the synthesis of a phosphoramidite suitable for incorporating into a DNA molecular beacon. The unprecedented addition of a dimethoxytrityl group to the heterocyclic ring system of the pC nucleobase is described.
Chapter 2 - Toward Multiple Labelling of PNA Oligomers by Sequential Azide-Alkyne Cycloaddition Reactions

2 Chapter Introduction

While PNA represents a promising framework for molecular beacons, the incorporation of fluorophore and quencher moieties into such MBs represents a challenge. The traditional approach, in the Hudson research group and others, has been to synthesize PNA monomers that incorporate these moieties. Such monomers are generally obtained via multi-step syntheses that can be time consuming and low yielding. Furthermore, characterization of their photophysical properties can sometimes yield disappointing results. A simpler approach to PNA MB derivitization, therefore, is desirable.

One method that has emerged as a possibility for PNA derivatization is the copper-catalyzed azide-alkyne cycloaddition, or CuAAC. First described simultaneously by Sharpless and Meldal in 2002, the CuAAC reaction is a relatively simple, regiospecific reaction that involves the covalent linking of a terminal alkyne with an azide group to form a stable triazole moiety.

R\text{N} = \text{N} \text{N} + \text{R'} \xrightarrow{\text{Cu(I) (cat)}} \text{R N} = \text{N} \text{N}\text{R'}

Scheme 3. General scheme for CuAAC.

This reaction belongs to the general class of what Sharpless himself described as “click chemistry”. In fact, CuAAC is all but synonymous with the term “click chemistry”; as such, CuAAC and “click” will be referred to interchangeably in this text.

Two common approaches are used to perform CuAAC: 1) a copper (II) catalyst is reduced to copper (I) \textit{in situ} by a reducing agent such as ascorbate, or 2) a copper (I) catalyst is employed in the presence of base, typically an amine such as diisopropylethylamine (DIPEA). The reaction is run at room temperature, preferably in solvents such as water, and often results in swift conversion of starting materials to the 1,3-cycloaddition product.
The CuAAC reaction has been previously explored as a method for derivatizing PNA oligomers using solid-phase (“on-resin”) reactions. Recently, the Hudson group published the synthesis of a simple Fmoc-protected PNA monomer containing an azide moiety. This PNA monomer can be easily incorporated into PNA peptides by automated or manual solid-phase synthesis, enabling the derivatization of oligomers by on-resin CuAAC with compounds containing terminal alkynes.

**Figure 9.** Azide-containing PNA monomer.

An obvious application of this chemistry is the incorporation of fluorophore and/or quencher moieties into PNA molecular beacons. In fact, current work in the Hudson group has included the synthesis of a quencher-free molecular beacon in which an alkyne-derivatized pyrene moiety is incorporated into PNA by on-resin CuAAC.

Another advance toward CuAAC-derivatized PNA molecular beacons was achieved in the Hudson group with the design and synthesis of an analog of the “universal quencher” 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL). This DABCYL analog contains an alkyne moiety that can serve as a “click partner” with the azide PNA monomer.

**Figure 10.** DABCYL analog alkyne I.
It remained to establish a library of novel “clickable” fluorophore derivatives that could also be incorporated into PNA via on-resin click chemistry. Such fluorophores could conceivably find utility beyond incorporation into molecular beacons; one potential application might be the labeling of proteins by attaching the fluorophores to noncanonical azide-containing amino acids using click chemistry.\textsuperscript{43}

Finally, a methodology was devised to enable the incorporation of both a quencher and fluorophore moiety into a PNA peptide via on-resin CuAAC. A major application of molecular beacons is the detection of single nucleotide polymorphisms (SNPs), owing to the selectivity of binding that can be tuned to recognize mismatches of a single base pair. As such, the proposed nuclebase sequence was designed to function as a probe for the Trp1282Ter mutation (W1282X) of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.\textsuperscript{44}

### 2.1 Proposed Synthetic Scheme for Molecular Beacon

A four-step methodology was devised for the synthesis of a PNA molecular beacon, involving two separate rounds of automated peptide synthesis and two separate manual on-resin CuAAC reactions.

Step 1 of the scheme is the synthesis of partial peptide (PNA 1) via standard Fmoc chemistry, incorporating the azide PNA monomer toward the C terminus. Step 2 is incorporation of quencher via on-resin CuAAC to yield PNA 2. Step 3 involves completion of peptide synthesis to yield the complete PNA sequence with a second azide monomer near the N terminus (PNA 3). Finally, step 4 is incorporation of alkyne-derivatized fluorophore via on-resin CuAAC to yield the complete MB (PNA 4).

This methodology, if successful, would represent a novel and relatively simple method for the derivatization of PNA oligonucleotides as molecular beacons. The alkyne-modified quencher and fluorophores can be easily synthesized, and can all function as “click partners” with the azide PNA monomer, representing a potential improvement over standard syntheses of molecular beacons incorporating modified fluorescent/quenching PNA monomers. While all three fluorophores synthesized have previously been described,\textsuperscript{45-47} two of the compounds – the
acridine and acridone derivatives – have not previously been reported for use as CuAAC “click partners”.

Scheme 4. Proposed methodology for synthesis of PNA molecular beacon.

2.2 Quencher and Fluorophores

2.2.1 DABCYL Alkyne Analog Quencher

Synthesis and characterization of the DABCYL analog alkyne 1 has previously been achieved in the Hudson group. Following the synthesis of an iodinated precursor, the terminal alkyne can be readily installed via Sonogashira coupling with TMS acetylene in excellent yields of up to 97%. TMS deprotection then affords the free DABCYL alkyne in quantitative yield.
2.2.2 Fluorophore I: Acridine

2.2.2.1 Rationale

The first fluorophore targeted for alkyne derivatization was acridine. This polycyclic aromatic compound is a well-characterized fluorophore\textsuperscript{48} that has also previously been reported as a DNA intercalating agent.\textsuperscript{49} A common derivative of acridine, 9-aminoacridine, is intensely fluorescent with a reported quantum yield of 0.99 in ethanol.\textsuperscript{50}

![Acridine and 9-aminoacridine](image)

**Figure 11.** Acridine (left) and 9-aminoacridine.

It was expected that a 9-aminoacridine derivative alkylated at N9 with a propargyl group would thus be both highly fluorescent and easily incorporated into azide-containing PNA via on-resin CuAAC.

![9-propargyl aminoacridine derivative II](image)

**Figure 12.** 9-propargyl aminoacridine derivative II.

2.2.2.2 Synthesis

A preparation method for alkylated 9-aminoacridines had previously been reported by Reisch and Gunaherath,\textsuperscript{45} and this method was employed for initial attempts at synthesizing compound 2. 9-Chloroacridine (initially purchased from Sigma-Aldrich, later synthesized according to a literature procedure\textsuperscript{51}) was fused with phenol at 110° C, and propargylamine HCl salt was added along with DIPEA to deprotonate the propargylamine salt *in situ*.
Scheme 5. Initial scheme for synthesis of compound II.

This method resulted in a mixture of products that was difficult to purify. Subsequent attempts to perform the reaction without added base enabled the isolation of a small amount of yellow solid which appeared to correspond to reported values for compound II by $^1$H NMR. However, this solid was acquired in low yield and also resisted purification.

Fresh propargylamine was purchased and the reaction was reattempted according to Reisch and Gunaherath’s conditions. In this instance the starting material was consumed quickly, but the product obtained was a bright yellow powder, highly fluorescent in solution, whose RF by TLC and $^1$H NMR spectrum did not conform to known values for the desired product. Initially it was believed that this compound might be acridone, a well-known byproduct of the hydrolysis of 9-chloroacridine; however, the $^1$H NMR spectrum, RF by TLC, and fluorescence of acridone did not correspond to this byproduct. Comparison of the $^1$H spectrum of 9-aminoacridine to that of the byproduct indicated that said byproduct was in fact 9-aminoacridine rather than acridone.

Given the unsatisfactory results obtained from Reisch and Gunaherath’s synthetic method, a gentler approach was attempted by stirring 9-chloroacridine with propargylamine in acetonitrile.

Scheme 6. Revised procedure for synthesis of HCl salt of compound II.
The reaction was sluggish at room temperature, but on heating the mixture to 50°C rapid formation of a yellow precipitate was observed. This solid was isolated via filtration and appeared by \(^1\)H NMR to be the hydrochloride salt of the desired acridine alkyne derivative. Yield of the product obtained was 70%. Thus this synthetic method represents an improvement on Reisch and Gunaherath’s procedure in yield, mildness of conditions, and ease of purification of the product.

The 9-aminoacridine alkyne was subjected to a CuAAC reaction with benzyl azide to verify its suitability as a CuAAC “click partner”. No reaction was observed when the click reaction was attempted with compound I using standard ascorbate/copper sulfate click conditions,\(^{53}\) so it was decided to reattempt the click reaction using a procedure originally developed using acetic acid, CuI and DIPEA in DCM.\(^{54}\) Because the acridine alkyne was itself an HCl salt, the acetic acid was omitted from the reaction conditions. This method proved successful, with starting material consumed in 0.5 hours as determined by TLC, and formation of the triazole “click product” confirmed by \(^1\)H NMR.

2.2.3 Fluorophore 2: 4-Nitrobenzo-2-oxa-1,3-diazole (NBD)

2.2.3.1 Rationale

The second fluorophore targeted for alkyne derivatization was 4-nitrobenzo-2-oxa-1,3-diazole (NBD).

![Figure 13. Structures of NBD-Cl and alkyne derived from NBD (compound III).](image)

The intense fluorescence of benzofurazan compounds with amine moieties at the 7 position was first reported in 1968.\(^{55}\) The ready reaction of the non-fluorescent 7-chloro derivative of NBD
with amines suggested applications in the fluorescent tagging of biological molecules such as amino acids.  

The NBD fluorophore has a reported emission maximum at 535 nm in EtOH$^{46}$ and may not be ideal as a FRET partner for a DABCYL analog. However, it has the interesting property of being highly environmentally sensitive. Notably, the intensity of its fluorescence is extremely solvent-dependent, with markedly higher fluorescence noted in organic solvents relative to polar solvents such as water; furthermore, a red shift in emission is noted with increasing polarity of solvent. $^{56}$ These qualities have led to extensive deployment of NBD fluorophores as tags for lipids and cholesterols. $^{57}$

Recently, the NBD fluorophore was also shown to have potential application as a DNA mismatch probe, when Thiagarajan et al. reported a probe system with NBD ligands capable of inserting themselves into a probe DNA strand containing an abasic site. $^{58}$ The covalent incorporation of an NBD moiety into a molecular probe might enable identification of abasic sites in nucleic acid targets.

### 2.2.3.2 Synthesis

Synthesis of the alkyne-derivatized NBD compound was achieved with much greater ease than that of the acridine derivative, using an established literature procedure. $^{46}$ NBD-Cl, a commercially available compound, was stirred with propargylamine in acetonitrile at room temperature, and the product was isolated in good yield via column chromatography.

![Scheme 7. Synthesis of compound III.](image-url)
A “test click” was performed with this compound and benzyl azide according to conditions already employed by Key and Cairo.\textsuperscript{46} The triazole product was isolated and its structure confirmed by \textsuperscript{1}H NMR, although the reaction also produced an insoluble dark solid.

2.2.4 Fluorophore 3: Acridone

2.2.4.1 Rationale

Acridone is a well-studied fluorophore with strong fluorescence emission in the 400-450 nm range. Like acridine, it has therapeutic applications as a DNA intercalator,\textsuperscript{59} and has also been reported as a base-discriminating fluorophore for the detection of SNPs.\textsuperscript{60} Acridone has been reported as forming a FRET pair with DABCYL.\textsuperscript{61}

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{acridone.png}
\caption{Acridone.}
\end{figure}

This compound was not originally targeted for alkyne derivitization. However, when the byproduct from attempts to synthesize compound \textbf{II} was believed to be acridone, the intense fluorescence of the byproduct prompted investigation into acridone and its properties. Apart from its potential for FRET pairing with DABCYL, acridone also has the interesting property of being quenched by guanine.\textsuperscript{61} This suggests the possibility of employing this fluorophore in a quencher-free PNA molecular beacon.

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{acridone_derivative.png}
\caption{N-propargyl acridone derivative \textbf{IV}.}
\end{figure}
2.2.4.2 Synthesis

The starting material, acridone, was readily synthesized according to a literature procedure.\textsuperscript{62} The alkynyl derivative was obtained by deprotonation of the amine with sodium hydride and alkylation with propargyl bromide.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=0.8\textwidth]{synthesis.png}};
\end{tikzpicture}
\end{center}

\textbf{Scheme 6.} Scheme for synthesis of compound \textbf{IV}.\textsuperscript{47}

Initial “test click” reactions with compound \textbf{IV} with previously used conditions\textsuperscript{53} resulted in no product formation, possibly due to the lack of solubility of the acridone alkyne in polar solvents. The acridone alkyne was successfully “clicked” to benzyl azide with CuI using acetonitrile as solvent/ligand,\textsuperscript{63} although a reaction time of two days was required for full consumption of starting material as determined by thin layer chromatography.

2.3 Fluorescence Studies – Fluorophores

Absorption/emission spectra, quantum yields, and molar extinction coefficients were obtained for compounds \textbf{II}, \textbf{III}, and \textbf{IV} in ethanol.
Table 1. Fluorescence data for fluorophores acquired in ethanol.

<table>
<thead>
<tr>
<th>Alkyne</th>
<th>$\lambda_{\text{max}}$ (absorbance)</th>
<th>$\lambda_{\text{max}}$ (emission)</th>
<th>Quantum yield</th>
<th>Molar absorptivity (L·M⁻¹·cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-aminoacridine (II)</td>
<td>400</td>
<td>490</td>
<td>0.09</td>
<td>8800 (411 nm)</td>
</tr>
<tr>
<td>NBD (III)</td>
<td>450</td>
<td>515</td>
<td>0.41</td>
<td>17379 (449 nm)</td>
</tr>
<tr>
<td>Acridone (IV)</td>
<td>395</td>
<td>410</td>
<td>0.76</td>
<td>10703 (396 nm)</td>
</tr>
</tbody>
</table>
The quantum yield of 0.09 for compound II was disappointing, especially relative to that of 9-aminoacridine. However, 9-aminoacridine has in fact been shown to undergo a marked decrease in fluorescence upon alkylation of the exocyclic amine group,\textsuperscript{64,65} possibly due to steric clashing of the bulky alkyl group disrupting planarity of the ring system.\textsuperscript{66} Compound III had a respectable quantum yield of 0.41, although this is lower than the reported literature value of 0.57. Of the three fluorophores, compound IV had the highest quantum yield at 0.76, comparable to literature values for acridone at 0.78.\textsuperscript{67}

2.4 Early Attempts Toward Molecular Beacon Synthesis

2.4.1 First Attempt

Synthesis of the initial 9-mer containing an azide residue near the C-terminus (PNA 1) was achieved on a 5 µmol scale via standard Fmoc chemistry using NovaSyn TGR rink amide resin with a substitution of 0.045 meq/g. A 3 mg portion of resin was cleaved using TFA/triethylsilane and the cleaved peptide was examined by liquid chromatography mass spectrometry (LC-MS). The concurrent LC trace of the cleaved peptide indicated that it was of sufficient purity to carry forward to the next step.

The first on-resin click reaction, using compound I as a “click partner” with the azide monomer, was attempted using conditions that had been previously been successful for on-resin click chemistry with this monomer.\textsuperscript{41}

After overnight reaction, a portion of the resin was again cleaved and checked by mass spectrometry – the LC trace indicated that the reaction was roughly 50% complete. Additional reagents were added and the reaction was continued. After 48 more hours of reaction the starting material was no longer visible in a sample of cleaved resin by LC-MS; however, the LC trace was increasingly “messy”, with other byproducts appearing to have formed. However, given that no starting material appeared to remain, this quencher-derivatized resin was carried forward to the next step. Automated synthesis of the full 18-mer (PNA 3) was attempted under the previously described conditions. However, on completion of the automated synthetic run, a sample of cleaved peptide was examined by LC-MS and it was determined that no discernible PNA 3 had formed. In fact the LC-MS spectra were nearly identical to those of the starting peptide, with the Fmoc groups on the peptide apparently still intact.
It was theorized that the long reaction time and numerous equivalents of reagents required to force the click reaction to completion might have resulted in chemical or mechanical degradation of the resin which rendered the second round of automated peptide synthesis unfeasible. Therefore, it was decided to explore alternative conditions for the on-resin CuAAC.

For testing purposes 500 mg of TGR-R resin was loaded with azide PNA monomer. The first alternative on-resin click procedure attempted was one developed by Jagasia et al. for the cyclization of peptides containing alkyne and azide residues via on-resin CuAAC. The procedure used DMSO and MeCN as the solvent mixture. The intended purpose of the DMSO was to swell the resin, but this solvent also aids in dissolving the alkyne, which is a further advantage as solubility had previously been an issue with test click reactions involving compound IV. Furthermore, MeCN can also function as a ligand in CuAAC, which may also aid the reaction. Finally, 2,6-lutidine was used as the base; lutidine is considerably less basic than DIPEA, with a pKa of 6.65, so the use of this base might also prevent the Fmoc group on the terminal residue from being removed by the amine base used for Cu(I)-mediated CuAAC.

A “test click” was performed using this procedure to couple compound 4 to the resin-bound azide monomer. The literature procedure was deviated from only in that a twofold molar excess of fluorophore was used (the original procedure was developed for 1:1 azide/alkyne equivalents). After overnight reaction the reaction appeared complete as verified by LC-MS. A second test reaction was performed, this time with a shorter reaction time of 4 hours; once again, the reaction appeared successful. Successful on-resin “test clicks” were performed using compounds I, II, and III. It is interesting to note that although the “test click” of the NBD fluorophore with benzyl azide had produced an insoluble byproduct, the on-resin test click proceeded cleanly with no byproduct observed. This procedure appears promising for the on-resin CuAAC steps involved in the four-step molecular beacon synthetic methodology.

2.4.2 Molecular Beacon Synthesis – Second Attempt

For the second attempt at molecular beacon synthesis, a shorter sequence (PNA 1a) was synthesized using using cross-linked ethoxylate acrylate (CLEAR)-amide resin preloaded with lysine to a substitution of 0.094 meq/g. While this substitution is considered high for PNA synthesis, CLEAR resin is designed for difficult peptide syntheses; it therefore was expected
that PNA synthesis would proceed without problems. In fact this seemed to be the case, with successful synthesis of PNA 6-mer verified by LC-MS.

Figure 17. PNA 6-mer 1a.

The DABCYL analog quencher alkyne was then installed on the peptide using Jagasia et al.’s conditions for on-resin CuAAC, again with a 2x molar excess of alkyne. After overnight reaction a portion of the resin was cleaved and the peptide was examined by LC-MS. The spectra confirmed the disappearance of the starting material and indicated a clean peak corresponding to the clicked product.

The resin containing the clicked peptide was returned to the automated synthesizer and the synthesis was continued. However, a sample of cleaved peptide examined by MS appeared to contain only acylated (“capped”) PNA, indicating that the second attempt at extending the peptide on the ABI automated synthesizer was thus also unsuccessful.

Figure 18. Comparison of LC traces at 260 nm for clicked PNA 1a (top) with clicked PNA 1.
2.5 Revised Synthetic Scheme

Following the failure of two separate attempts to extend “clicked” resin on the ABI synthesizer, it was proposed that this was not an ideal method for completing synthesis of the full peptide. Therefore, a revised synthetic scheme was devised.

This procedure begins with automated synthesis of PNA 1b, including the entire target sequence, up to the second azide monomer. This longer peptide would undergo the first round of on-resin CuAAC; the second azide monomer and the second lysine residue would then be installed by manual solid phase peptide coupling, and the second on-resin “click” reaction could then be performed to yield PNA 4. While slightly more complex than the initial scheme, this methodology would have the benefit of allowing troubleshooting when coupling the final residues to the first clicked peptide.

Scheme 8. Revised scheme for molecular beacon synthesis.
2.6 Toward Synthesis of Molecular Beacon by Revised Scheme

2.6.1 First Attempt

The first attempt at synthesis of PNA 1b was performed on the ABI synthesizer as previously described, again using CLEAR resin preloaded with lysine to a substitution of 0.094 meq/g. Following synthesis, the cleaved peptide was again examined using LC-MS. While the full peptide was identifiable, the spectra also indicated the presence of an acylated peptide only six residues long. Both product masses were found under a single peak both in the MS chromatograph and the LC trace, which made it difficult to determine the ratio of full peptide to 6-mer. In the hope of assessing the relative amount of 16-mer compared to the 6-mer byproduct, a portion of resin was cleaved and run on a polyacrylamide gel according to a procedure previously developed in the Hudson group. 70

Figure 19. Separation of PNA by gel electrophoresis. Lane 1 contains a PNA sequence 14 monomers in length, used as a standard; lane 2 contains PNA from attempted synthesis of PNA 2A. (Smear below band is indicative of unpurified PNA.)
This gel showed two bands, of similar intensity, that had run a very similar distance through the gel. The closeness of the two bands, despite the disparity in molecular weight between the two identified PNA sequences, may be explained by the similar charge-mass ratios of the two sequences in acidic buffer.²⁰

An on-resin CuAAC reaction was performed with DABCYL analog alkyne I in the hope that the clicked peptides would separate out more readily and their relative amounts would be discernable via LC-MS. However, as with the unclicked peptide, the clicked peptides were both found under the same peak in both the chromatograph and LC trace. A second gel was run using the clicked peptide, with a near identical result (data not shown).

Excision of the two major bands from the gel and extraction of the PNA yielded very poorly concentrated samples – in fact, only the 6mer could be discerned by LC-MS. It seemed apparent that the 6-mer was a major product of the synthesis, possibly due to aggregation of the PNA on the highly substituted resin. It is interesting to note that the 6-mer corresponds in length to the acylated product obtained from the attempt to extend the clicked PNA 6-mer on the peptide synthesizer. This suggests that the failure of the second round of automated synthesis may not be due to degradation of the resin or impurities from the click reaction, but may have also been caused by aggregation of the PNA on the resin.

2.6.2 Second Attempt: Poly-T Sequence

Given the difficulties attendant on synthesizing the CFTR probe sequence, it was decided to attempt the molecular beacon synthesis using a simpler “model” sequence consisting largely of thymine PNA monomers:

![Figure 20. Sequence of poly-thymine model PNA sequence 1c.](attachment:figure20.png)
Automated synthesis of the poly-T sequence proceeded as previously described. However, MS/UPLC analysis of the synthesized peptide once again showed that while the full peptide could be identified, the major product appeared to be a truncated sequence – in this case, a 7-mer. A second attempt at automated synthesis of PNA 1c, this time with extremely careful drying and handling of all PNA monomers and reagents, yielded a near-identical result.

In order to eliminate the possibility that the synthetic difficulties were caused by malfunctioning of the ABI synthesizer, a fully manual solid-phase synthesis of the poly-T sequence was attempted, again on a 5 µmol scale using the CLEAR resin substituted to 0.094 meq/gram. Coupling efficiency was monitored by Kaiser test. Initially, the synthesis appeared to be proceeding well, but after the sequence had grown to seven monomers, the Kaiser test indicated an inefficient coupling. It appeared that the manual synthesis was failing at the same point as the automated synthesis, possibly due to aggregation on the resin. Two further attempts to couple more T monomer to the resin, using DMSO as a solvent as recommended to disrupt aggregation, did not visibly reduce the amount of free amine discernible by Kaiser test. A portion of resin was cleaved and the peptide examined by LC-MS. A prominent peak could be detected for the desired 8-mer, but peaks for both Fmoc-protected and Fmoc-unprotected 7mer were also found. The failure of the resin deprotection step, normally achieved quite readily by shaking the resin with 20% piperidine in DMF, is further indicative of aggregation of the poly-T peptides on the resin. At this point, time constraints necessitated a halt in the work.

2.7 Conclusion and Outlook

A synthetic scheme was devised for the synthesis of a PNA molecular beacon differentially labeled with a fluorophore and quencher by on-resin CuAAC. A library of three simple alkyne-derivatized fluorescent molecules was synthesized, their fluorescence properties were characterized, and their viabilities as on-resin “click partners” with an azide PNA monomer were demonstrated via a clean, fast, and efficient on-resin click procedure. It is unfortunate that technical difficulties impeded progress of the full synthesis of the molecular beacon. However, immediate future work will involve investigation into resins more suitable for PNA peptide synthesis, as it seems apparent that many synthetic difficulties can be attributed to aggregation of PNA on the resin used despite claims that said resin is useful for the synthesis of difficult
sequences. This will likely enable successful synthesis of PNA 1a, or a simpler test sequence, and exploration of the synthetic methodology can then continue.

2.8 Experimental

General Remarks

All chemicals were obtained from commercial sources and used without further purification except where noted. NMR spectra were acquired on a Varian Mercury 400 instrument. Chemical shifts are reported in parts per million (δ), were measured from tetramethylsilane (0 ppm), and are referenced to the residual proton in the deuterated solvent: acetone – d₆ (2.05 ppm), CDCl₃ (7.26 ppm), DMSO – d₆ (2.49 ppm) for ¹H NMR and CDCl₃ (77.0 ppm) and DMSO – d₆ (39.5 ppm) for ¹³C NMR spectroscopy. Multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br s (broad singlet). Coupling constants (J) are reported in hertz (Hz). High resolution mass spectra (HRMS) and low resolution mass spectra were obtained using electron impact (EI) or electrospray ionization (ESI) methods.

Preparation of 9-chloroacridine:⁵¹

N-phenylanthranilic acid (10.0 g, 47 mmol) was placed in an RBF and POCl₃ (50.0 g, 326 mmol) was added via slow pour. The mixture was placed in an oil bath and slowly heated to 90°C. The mixture was cooled to room temperature, then reheated to 135°C and refluxed for two hours. Excess POCl₃ was removed by vacuum distillation and the dark brown mixture was cooled to room temperature. Concentrated aqueous ammonia (50 ml), chloroform (50 ml), and ice were combined in a beaker and poured into the flask containing the crude product. The mixture was stirred until it became cloudy and orange-brown. The mixture was partitioned in a separatory funnel and the organic layer was collected. The aqueous layer was washed with chloroform (3 x 25 ml) and the organic extracts were dried with Na₂SO₄. Solvent was removed to yield the product as a light brown solid that was used without further purification (7.5 g, 75%). ¹H NMR (DMSO-d₆) δ: 8.42 (d, J = 8.6 Hz, 2H) 8.22 (d, J = 9.0 Hz, 2H) 7.95 (m, 2H) 7.80 (m, 2H). – characterization conformed to reported values.

Preparation of 9-propargylaminoacridine • HCl (compound II):
9-chloroacridine (0.500 g, 2.3 mmol) was dissolved in anhydrous acetonitrile. Propargylamine (0.258 g, 4.6 mmol) was added and the mixture was heated to 50°C, then stirred for 24h. The bright yellow precipitate was collected via filtration to yield compound II as an HCl salt (0.441 g, 70%). $^1$H NMR (DMSO-$d_6$) $\delta$: 10.42 (bs, 1H), 8.74 (d, $J = 8.7$ Hz, 2H), 8.02 (m, 4H), 7.62 (m, 2H), 4.88 (s, 2H), 3.68 (s, 1H). HRMS (EI) calcd. for C$_{16}$H$_{12}$N$_2$: 232.1000. Found: 232.0991.

“Test click” of compound II with benzyl azide: $^{54}$

DCM (5 ml) and DIPEA (0.0232 g, 0.18 mmol) were combined in an RBF and CuI (0.0069 g, 0.036 mmol), compound II (0.050 g, 0.18 mmol), and benzyl azide (0.0273 g, 0.2 mmol) were added to the flask. The mixture was stirred for 0.5 hours, then filtered and the filtrate was concentrated under vacuum. The resulting orange-yellow solid was precipitated in toluene and collected by filtration (0.0454 g, 66%). $^1$H NMR (DMSO-$d_6$) $\delta$: 8.60 (d, $J = 8.6$ Hz, 2H), 8.31 (s, 2H), 7.97 (m, 2H), 7.87 (d, $J = 8.6$ Hz, 2H), 7.51 (t, $J = 7.20$ Hz x2, 2H), 7.35 (m, 2H), 7.24 (d, $J = 8.2$, 2H), 5.61 (s, 2H), 5.34 (s, 2H).

Preparation of 4-propargylamine NBD (compound III): $^{46}$

NBD-Cl (0.200 g, 1.0 mmol) was dissolved in dry acetonitrile. Propargylamine (0.138 g, 2.5 mmol) was added and the dark green mixture was stirred at RT for 1.5 hours. Solvent was removed via vacuum and the black crude solid was purified via FCC on silica gel (3:1.5 hexanes/EtOAc) to yield compound III (0.178 g, 81%). $^1$H NMR (CDCl$_3$) $\delta$: 8.54 (d, $J = 9$ Hz, 1H), 6.36 (s, 1H), 6.34 (s, 1H), 4.31 (m, 2H), 2.43 (s, 1H) – characterization conformed to reported values.

“Test click” of compound III with benzyl azide: $^{46}$

CuSO$_4$ (0.003 g, 0.018 mmol), sodium ascorbate (0.005 g, 0.018 mmol), and compound III (0.020 g, 0.09 mmol) were dissolved in a 1:1 mixture of MeOH:H$_2$O (5 ml). Benzyl azide was added dropwise and the mixture was stirred at RT for six hours. Solvent was removed and the crude solid was partitioned in CHCl$_3$/H$_2$O and the organic phase was washed with H$_2$O. The organic phase was collected and dried with Na$_2$SO$_4$ and solvent was removed to yield the product as a yellow-orange solid (0.0093 g, 29%). $^1$H NMR ((CD$_3$)$_2$CO) $\delta$: 8.53 (d, $J = 9$ Hz,
2H), 8.09 (s, 2H), 7.34 (d, J = 3.1, 8H), 6.62 (d, J = 9, 2H), 5.62 (s, 4H) 4.93, br s, 4H), 3.31 (d, J = 5.5, 1H), 2.84 (s, 9H), 2.81 (s, 3H).

Preparation of acridone:

N-phenylantranilic acid (2.0 g, 9.4 mmol) was placed in an RBF and concentrated aqueous H₂SO₄ (5 ml) was added dropwise. The mixture was stirred until all solids dissolved and the red-brown solution was heated to 100°C in an oil bath and stirred for 4 hours. The solution was poured slowly into boiling water, and this mixture was carefully boiled for five minutes. The mixture was removed from heat and a pale yellow solid was collected by filtration. The solid was transferred to a flask containing boiling aqueous K₂CO₃ solution. This mixture was carefully boiled for five minutes and a yellow solid was collected by filtration and washed with H₂O. The yellow solid was coevaporated with toluene to yield the product (1.2 g, 66%). ¹H NMR (DMSO-d₆) δ: 11.75 (s, 1H) 8.23 (dd, J = 8.2 Hz, 1.6 Hz, 2H) 7.73 (m, 2H) 7.54 (d, J = 8.6, 2H) 7.26 (m, 2H). – characterization conformed to reported values.

Preparation of 10-propargylacridone (compound IV):

Acridone (0.500 g, 2.6 mmol) was suspended in 15 ml anhydrous DMF. Sodium hydride (60% suspension in mineral oil, 0.112 g, 2.8 mmol) was added in portions and the mixture was heated to 50°C and stirred for 0.5 hours. The yellow-green fluorescent solution was cooled to RT and propargyl bromide (0.389 g, 3.1 mmol) was added. The mixture was stirred for 4 h and poured into 40 ml H₂O. The precipitate was collected via filtration and recrystallized from anhydrous EtOH to yield compound IV as pale yellow crystals (0.368 g, 61%). ¹H NMR (DMSO-d₆) δ: 8.35 (m, 2H) 7.88 (m, 2H) 7.38 (m, 2H) 5.37 (d, 2H, J = 2.4 Hz) 3.43 (t, J = 2.4 Hz, 1H). ¹³C NMR (CDCl₃) δ: 141.62, 134.05, 127.83, 122.64, 121.75, 114.51, 73.84, 36.79.

“Test click” of compound IV with benzyl azide:

MeCN (5 ml) was placed in an RBF and benzyl azide (0.0120 g, 0.09 mmol) was added. Compound IV (0.020 g, 0.09 mmol) and CuI (0.0017 g, 0.009 mmol) were added. The mixture was stirred at RT for 48 hours; solvent was then removed and a light yellow powder was collected by filtration after being washed with H₂O (0.0123 g, 37%). ¹H NMR (DMSO-d₆) δ:
8.35 (d, J = 6.6 Hz, 2H) 8.21 (s, 1H) 7.95 (d, J = 8.6 Hz, 2H) 7.82 (m, 2H) 7.32 (m, 8H) 5.81 (s 2H) 5.53 (m, 2H).

**General procedure for “test click” reactions of alkynes with resin-bound PNA azide monomer:**

A mixture of DMSO (1 ml) and acetonitrile (4 ml) was degassed by bubbling with N₂ gas for 5 min, after which CuI (0.005 g, 2.5 µmol) was added. TGR-R resin downloaded with azide PNA monomer (0.050 g, 0.01 mmol) was placed in a manual peptide synthesis vessel, followed by alkyne (0.02 mmol). CuI solution (2 ml) and 5 µl 2,6-lutidine were added to the vessel and the mixture was degassed by bubbling with N₂ gas for 3 minutes. The vessel was flushed with N₂, sealed, and shaken overnight. The resin was then washed 4x with DCM/DMF.

**Synthesis of PNA 8-mer 1:**

The PNA 8-mer containing azide monomer was synthesized on a 5-µmol scale on an Applied Biosystems 433A peptide synthesizer. Resin used was NovaSyn TGR (rink amide) resin preloaded with Boc-lysine (substitution 0.045 meq/g).

**Synthesis of PNA 2 (incorporation of DABCYL analog quencher I into PNA 8-mer 1 via on-resin CuAAC):**

Resin containing PNA 1 (0.073 g, 3 µmol) was placed in a manual peptide synthesis vessel with DABCYL analog I (0.008 g, 30 µmol). DIPEA (0.058 g, 300 µmol), THF (2.5 ml), and pyridine (1.5 ml) were added to the vessel, followed by CuI (0.057 g, 300 µmol). The vessel was wrapped in aluminum foil and shaken for 3 days, with addition of 30 µmol of compound I and 300 µmol CuI each day. The resin was then washed with anhydrous DCM and anhydrous DMF (3 cycles).

**Synthesis of PNA 6-mer 1a:**

PNA 6-mer 1a was synthesized as previously described. Resin used was CLEAR-amide resin preloaded with Boc-lysine (substitution 0.094 meq/g).
Synthesis of PNA 2a (incorporation of DABCYL analog quencher I into PNA 6-mer 1a via on-resin CuAAC):

A mixture of DMSO (1 ml) and acetonitrile (4 ml) was degassed by bubbling with N\textsubscript{2} gas for 5 min and CuI (0.005 g, 2.5 \textmu mol) was added. Resin containing PNA 6-mer 1a was placed in a manual peptide synthesis vessel, followed by compound I (0.0023 g, 0.01 mmol). 2 ml of the CuI solution and 5 \textmu mol 2,6-lutidine were added to the vessel and the mixture was degassed by bubbling with N\textsubscript{2} gas for 3 minutes. The vessel was flushed with N\textsubscript{2}, sealed, and shaken overnight.
Chapter 3 – Toward Synthesis of DABCYL Analog Pyrrolocytidine Phosphoramidite

3 Chapter Introduction

The universal quencher DABCYL has had a long and robust history as a quencher in FRET pairs, notably in molecular beacons. The Hudson group had previously entered the realm of quenching nucleic acid analogues with the synthesis and evaluation of a PNA monomer containing the DABCYL diarylazo moiety.\textsuperscript{33,74}

![Figure 21 PNA monomer containing azobenzene moiety in place of a standard nucleobase.](image)

The design and successful synthesis of the DABCYL alkyne analog 1, previously described in this report as a “clickable” quencher for labeling of azide-containing PNA, suggested an application in the Sonogashira-cyclization methodology previously employed in the Hudson group for the synthesis of fluorescent pyrrolocytidine (pC) base analogs.\textsuperscript{75,76}

Incorporating the DABCYL diarylazo moiety into a cytidine analog would result in a custom nucleotide capable of quenching fluorescence via FRET while retaining the Watson-Crick face required for successful base pairing with guanine.
The free nucleoside had previously been synthesized in sufficient quantity for characterization of structure and photophysical properties. In fact, the azo pC nucleoside was shown to have molar extinction coefficients in ethanol at 260 and 445 nm superior to those of DABCYL. It was thus desirable to synthesize this compound in greater quantities and to complete its chemical transformation to a phosphoramidite suitable for incorporation into DNA oligonucleotides.

3.1 Phosphoramidite Chemistry

Developed in the early 1980s, the phosphoramidite method is a popular method for the solid-phase synthesis of custom DNA oligonucleotides. Synthesis proceeds in the 3'-to-5' direction by sequential coupling of phosphoramidite nucleobases to a solid support, often pre-derivatized with a DMT-protected nucleobase. Each coupling follows a 4-step process:

1. Removal of the DMT protecting group from the 5'-OH using acid, commonly 3-5% trichloroacetic acid (TCA) w/v in DCM.
2. Coupling of the resin-tethered nucleobase to the incoming phosphoramidite using an activator such as tetrazole.
3. Capping of any unreacted sites on the resin using an acetylating agent, commonly acetic anhydride.
4. Oxidation of the P(III) phosphite triester linkage to the more stable P(IV) phosphotriester using I₂ in H₂O.

Figure 22. Pyrrolocytidine-based nucleoside V incorporating DABCYL analog alkyne I.
When synthesis is complete, the oligonucleotide is cleaved from the resin using concentrated aqueous ammonia. This step also results in the removal of protecting groups such as benzoyl on the exocyclic amine groups of C and A, and the β-cyanoethyl group from the phosphate.
In order to be incorporated into oligonucleotides by phosphoramidite chemistry, DNA nucleotides must undergo two modifications: the 5'-OH must be protected by a DMT group, and the phosphoramidite group must be introduced at the 3'-OH.

### 3.2 Methodologies for Pyrrolocytidine Synthesis

Two synthetic methodologies have previously been deployed in the Hudson group for the synthesis of pC phosphoramidites. The major difference between the two routes lies with the iodinated nucleobases used as starting material: 5-iodouridine and 5-iodocytidine.

**Iodocytidine Route**

**Iodouridine Route**

**Scheme 10.** Cytidine and uridine routes for synthesis of pyrrolocytidines.
The uridine route requires a conversion of the furanouridine ring to pyrrolocytidine by an aminolysis reaction with liquid NH$_3$. This atom conversion is necessary due to the fact that the furanouracil moiety does not retain the W-C base pairing capabilities of cytosine with guanine. In fact, because N3 is deprotonated during ring formation, the furanouracil moiety loses its capability to form two hydrogen bonds with adenine, the natural W-C base pairing partner of uracil.

**Figure 23.** Hydrogen bonding capabilities of a) furanouracil-guanine, b) furanouracil-adenine, and c) pyrrolocytosine-guanine base pairs. R = H or alkyl substituent.

Despite the necessity for the aminolysis, the uridine route has several major benefits relative to the cytidine route. First, uridine is less costly than cytidine. Also, prior to Sonogashira coupling the exocyclic amine of cytidine must be benzoyl protected. In order to ensure selective benzoyl protection of the amine, the hydroxyl groups on the ribose sugar ring must first be protected with acyl or silyl ether protecting groups. Uridine contains no exocyclic functional groups that require protection, bypassing the need for both rounds of protecting group chemistry.

### 3.3 Toward Synthesis of DABCYL pC Phosphoramidite – Uridine Route

Synthesis of free nucleoside V had previously been achieved using cytidine as the starting point. However, it was decided to attempt synthesis of the phosphoramidite following the uridine route, which had been previously used successfully in the group to synthesize 6-phenylpyrrolocytidine.$^{75}$
Scheme 11. Scheme for synthesis of DABCYL pC phosphoramidite V-4 by uridine route.

DMT protection of 5-iodocytidine proceeded according to conditions previously described.\(^7^5\) The next step in the synthesis was Sonogashira coupling of the DABCYL analog alkyne I to 5-iodocytidine, followed by cyclization. Previously in the Hudson group, such couplings had been accomplished by a one-pot, two-step methodology. However, while the Sonogashira coupling proceeded readily, with disappearance of the starting material by TLC after overnight reaction at room temperature, attempts at performing the cyclization via addition of base/additional CuI and heating the mixture resulted in a “messy” mix of products as determined by TLC. It was decided, thus, to reattempt the Sonogashira coupling and isolate/purify the coupled alkyne product before attempting cyclization.
Scheme 12. Revised scheme for cyclization to furanouridine V-2.

The second Sonogashira coupling proceeded as before, and a bright orange solid was isolated following workup. However, while previous work in the Hudson group had indicated that furano ring formation would proceed rapidly under these conditions, the cyclization of the DABCYL analog-coupled uridine proved sluggish.

An initial attempt to cyclize to the furanouridine using AgNO₃ and acetone, conditions originally described by Agrofolio and previously reported in the Hudson group as successful in the synthesis of phenylfuranouridine, was unsuccessful. The starting material was reclaimed and the cyclization reaction set up using excess triethylamine, methanol, and CuI. After three days at 50°C and addition of more CuI the starting material was reduced but had not disappeared by TLC. Additionally, a new spot appeared by TLC that had a slightly higher RF and was slightly deeper red in colour than the spot for the desired product.

At this time the reaction was stopped and workup proceeded; the furanouridine product was isolated via column chromatography, but in moderate yield (44%). Fractions corresponding to the new spot were isolated as well, but an ^1^H NMR spectrum acquired for the resulting solid seemed to indicate a mixture of products. This spot likely corresponds to the cis isomer of the azo moiety. This isomerization is likely thermally promoted in this instance, as the reaction is run under dark conditions. The mixture of product peaks visible by NMR may be a result of the cis isomer reverting to the more stable trans isomer under visible light.
The next step was one that had proved challenging in previous syntheses: atom exchange via aminolysis to convert the furanouridine to pyrrolocytidine. This had been previously accomplished by stirring the furanouridine with condensed liquid NH₃ and methanol in a pressure vessel. However, due to concerns regarding a lack of solubility of the DABCYL analog furanouridine in methanol, it was decided to attempt the aminolysis in neat NH₃.

Initially it appeared that the reaction had proceeded, with disappearance of the starting material by TLC achieved after overnight reaction at room temperature. However, following purification, it appeared by MS that the major product of the reaction had a mass of 795.3729. This mass may correspond to this structure, which has the molecular formula C₄₀H₄₆N₆O₇ and a calculated mass of 795.3744:

![Proposed structure of major product of aminolysis reaction](image)

**Figure 24.** Proposed structure of major product of aminolysis reaction. The less stable enol tautomer is depicted for clarity.

It would appear that while the ammonia was able to attack and open the furano ring, the lack of an additional proton source – normally provided by the methanol – prevented the elimination of H₂O and the reaction thus stalled.

Given the small amount recovered of this undesirable product and due to time constraints, it was decided to attempt the phosphoramidite synthesis via the cytidine route.
3.4 Attempted Synthesis of DABCYL Analogue Pyrrolocytidine Phosphoramidite – Cytidine Route

An advanced cytidine intermediate, courtesy of Dr. Filip Wojciechowski, was located with the exocyclic NH$_3$ protected by a benzoyl group and the 5' and 3' hydroxyls bearing tert-butyl dimethylsilane (TBDMS) protecting groups.

Scheme 13. Proposed scheme for synthesis of DABCYL pC phosphoramidite V-4 by cytidine route.

In contrast to the uridine route, Sonogashira coupling and cyclization on the protected cytidine substrate were accomplished in tandem, with complete conversion of starting material as determined by TLC after two days of reaction at 50°C. Benzoyl deprotection is usually achieved in the same pot, but as with the attempted one-pot cyclization of the uridine intermediate, an attempt to perform the second reaction without previous purification resulted in an intractable
mixture of products. Therefore, it was once again decided to isolate the product of the first step of the reaction before attempting the deprotection.

Scheme 14. Revised scheme for benzoyl deprotection to yield compound V-6.

Following isolation of the benzoyl protected pyrrolocytidine intermediate, benzoyl deprotection was achieved by reaction with anhydrous EtOH/triethylamine, followed by removal of the TBDMS groups using NEt$_3$ • 3HF to afford the free nucleoside V in good yield (85% over three steps).

The next step was the DMT protection of the nucleoside. Great care was taken during setup of the reaction – anhydrous pyridine was further dried over sieves, triethylamine was also dried over sieves, and the starting material was coevaporated with pyridine and dried under high vacuum to prevent water contamination that could hydrolyze the DMT-Cl tritylating reagent. However, the tritylation reaction appeared sluggish, with persistence of starting material by TLC following 24 h of reaction. Catalytic AgNO$_3$ and additional DMT-Cl were added to the reaction mixture according to a procedure reported by Ogilvie, but this appeared to have no effect on the reaction. Finally the reaction was halted. Following basic workup, the product was purified by column chromatography in a moderate yield of 50%, but affording an adequate amount of product to proceed to the final step in phosphoramidite synthesis.

$^1$H NMR seemed indicative of formation of the desired product, with the methoxy and aromatic peaks appearing in the spectrum with proper integration. Furthermore, the exact mass of the
product as measured by MS was very close to the calculated mass (calculated 777.3401; found 777.3431).

Synthesis of the phosphoramidite was attempted following conditions previously employed in the Hudson group. Once again, synthesis appeared to proceed as usually observed for this procedure, with disappearance of starting material in three hours and a new spot appearing by TLC. Following purification, the $^1$H NMR spectrum of the orange solid obtained again appeared to correspond to the desired product. However, the $^{31}$P NMR spectrum did not show the customary pair of singlets in the 130-150 ppm range corresponding to the two diastereomers of the phosphoramidite, showing only a broad signal at approximately 138 ppm. It was theorized that the broad signal might be a result of the large, “floppy” phosphoramidite molecules tumbling slowly in solution, or caused by the presence of cis-trans azo isomers complicating the spectrum.

A MALDI MS spectrum was acquired for compound V-4. While the spectrum was not “clean”, a peak was observed at 975.8, close to the expected mass of 975.4. The lack of prominence of the peak in the spectrum was attributed to the instability of the phosphoramidite compound in the matrix.

Based on TLC and spectral evidence, it appeared that phosphoramidite V-6 had been successfully synthesized.

### 3.5 Molecular Beacon Design

Two oligonucleotide sequences were conceived, again targeting the the Trp1282Ter mutation (W1282X) of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.

**Table 2.** Proposed molecular beacon oligonucleotide sequences. Q = quencher (compound V-4), F = fluorophore (phenyl pC). Vertical lines indicate the probe sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-1</td>
<td>5'-Q-G-C-G-A-C</td>
</tr>
<tr>
<td>MB-2</td>
<td>5'-Q-G-C-G-A-G</td>
</tr>
</tbody>
</table>
The stem sequences were designed based on recommendations for probe design by Dr. Sanjay Tyagi\textsuperscript{81} and examined using the Zuker DNA folding program to verify that they would not be likely to form secondary structures that would disrupt stem hybridization.\textsuperscript{82} The shorter oligonucleotide, MB-1, was designed to hybridize to target sequences already available; as 14 nucleotides is slightly shorter than the average probe sequence of 15-30 nucleotides,\textsuperscript{81} a slightly longer probe sequence was envisioned as well (MB-2).

It remained only to verify the viability of phosphoramidite V-4 to be incorporated into an oligonucleotide before synthesis of the full molecular beacon sequences could begin.

### 3.6 Attempted Incorporation of Phosphoramidite into DNA Oligonucleotide

To test the coupling efficiency of compound V-6, a T-5 oligonucleotide was synthesized using standard phosphoramidite chemistry using commercially available thymidine phosphoramidites on an Applied Biosystems International 392 DNA/RNA synthesizer. A manual coupling of compound V-4 to this sequence was attempted. MS analysis of the product, however, indicated that no coupling had occurred. The synthesis was reattempted and the product examined by MS. Once again, only detritylated T-5 DNA was visible by MS.

Given the complete failure of the phosphoramidite coupling, and due to conflicting spectral evidence of product formation, it was decided to attempt a “test phosphitylation” with DMT-protected thymidine monomer to ensure the viability of the reagents and procedure. The reaction was set up using conditions identical to those used in the phosphitylation of compound V-3. The reaction was monitored by TLC, using commercially available thymidine phosphoramidite as a standard. The starting material appeared to be fully converted to product in two hours; following workup and purification conditions identical to those used for the DABCYL pC phosphoramidite, a $^{31}$P NMR spectrum was acquired for the T phosphoramidite. The spectrum contained the expected two strong, sharp singlets at 148 ppm. Furthermore, the spectrum contained a singlet near 139 ppm, very close to the broad singlet observed in the $^{31}$P NMR spectrum of the DABCYL pC phosphoramidite. This suggests that the signal in both spectra is caused by an impurity, likely the 2-cyanoethyl methyl diisopropylphosphoramidite.
generated by substitution of the chloride for a methoxy group on the phosphoramidite reagent during quenching of the reaction with methanol.

**Figure 25.** Comparison of $^{31}$P NMR spectra for DABCYL pC (left) and thymidine (right) phosphoramidites.
Figure 26 Detail of $^{31}$P NMR spectrum of thymidine phosphoramidite showing pair of singlets.

Given that the reagents and method used for phosphitylation were demonstrably viable, it appeared that the difficulties met during installation of the phosphoramidite group on compound V-4 using identical conditions indicated an intrinsic problem with the DMT-protected DABCYL pC nucleoside. In order to bypass the need for the phosphoramidite derivative, it was decided to directly couple compound V-4 to amine-functionalized controlled glass pore (CPG) resin according to a literature procedure. $^{83}$

Loading determination of the resin was attempted by trityl cation assay, but very little orange trityl cation was observed in acidic solution, and the loading value determined was extremely low – 2 µmol/g instead of the expected 30–40 µmol/g. A deprotection was attempted in neat trifluoroacetic acid, again with very little detritylation observable by colour. Samples of resin that had been treated with both 3% TCA in DCM and neat TFA were heated in concentrated
aqueous ammonia to liberate the nucleoside from the resin. In both cases, examination of the cleaved nucleoside by MS indicated that the trityl group was still attached (mass found: 777.3368. Mass of compound V-3: 777.3431). Given that the trityl group is highly acid labile, with deprotection normally occurring in 3% TCA on the order of seconds, this was a puzzling result.

### 3.7 Addition of Trityl Group to Heterocycle

The extreme and unexpected stability of the trityl protecting group to acid hydrolysis appeared indicative of the formation of a stronger bond during the tritylation reaction – perhaps even a carbon-carbon bond. As it turned out, this strange result was not without precedent in the Hudson group. A previously synthesized fluorescent pC nucleobase containing a furan ring had also resisted DMT deprotection.

![Figure 27. Structure of furan-substituted pC VI.](image)

A closer examination of the $^1$H NMR spectrum of compound V-3 indicated that the proton signal corresponding to H6 was not present. Previously, it had been believed that this signal was overlapping with the aromatic proton signals. An HSQC 2D NMR spectrum was acquired for compound V-3, but as a correlation could not be positively identified for either the C6 or pyrrolo carbons/protons, the result was considered inconclusive.

The $^1$H NMR spectrum for compound VI also lacked the H6 proton signal. It would seem in both cases that the trityl group had added to C6 of the cytidine ring, likely via electronic aromatic substitution (EAS) of the highly electron-rich aromatic system.
Scheme 15. Proposed mechanism for the addition of the dimethoxytrityl group to the pC heterocycle.

This would account for the unusual $^{31}$P spectrum of the product of the phosphoramidite reaction, as the failure to protect the 5' OH group with the DMT group would enable installation of the phosphoramidite group at that position, as well as the 3' OH group; this would also account for the failure of the DABCYL cytidine “phosphoramidite” to couple with DNA to give the desired product.

An attempt is ongoing to obtain a crystal of compound V-3 to unambiguously determine its structure via X-ray crystallography. At any rate, it seemed apparent that synthesis of phosphoramidite V-3 cannot be achieved by the conventional cytidine-based pC synthetic route.

3.8 Conclusion and Outlook

The unexpected difficulties that arose during synthesis of DABCYL pC phosphoramidite V-4 unfortunately precluded incorporation of the compound into DNA and studies into its utility as a quencher. However, given the potential demonstrated by the free nucleoside V in quenching studies, an alternative synthetic approach is merited. Future work will include a revisiting of the uridine route, with improved conditions for the atom exchange/aminolysis reaction, and exploration of alternative protecting group strategies for the cytidine route that would permit installation of the DMT group before Sonogashira coupling of the DABCYL analog alkyne to
iodocytidine/formation of the pC ring. Furthermore, the unprecedented attachment of the DMT group to the heterocycle by EAS, which appears to affect a small subset of electron-rich pyrrolocytidines, will be the subject of further study.

3.9 Experimental

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-5-iodouridine (V-1)

5-iodouridine (2.124 g, 6 mmol) was dissolved in anhydrous pyridine (45 ml) and Et₃N (6 ml) was added. The solution was brought to 0°C. 4,4'-dimethoxytrityl chloride (2.642 g, 7.8 mmol) was dissolved in 4 ml anhydrous pyridine. This solution was cooled to 0°C, then added dropwise to the 5-iodouridine solution. The mixture was brought slowly to RT and stirred overnight. The reaction mixture was quenched with MeOH (2 ml), then diluted with DCM and washed with 0.5 M NaHCO₃ (4 x 25 ml). The organic extracts were dried over Na₂SO₄ and the solvent was removed. The residue was purified by FCC eluting with 80:15:5 DCM:Et₂O:Et₃N and the product was isolated as a white foam (1.899 g, 48%).

1H NMR (400 MHz, CDCl₃) δ:
8.13 (s, 1H), 7.44-7.22 (m, 10H), 6.89-6.83 (m, 4H), 6.35-6.30 (m, 1H), 4.57-4.52 (m, 1H), 4.09-4.06 (m, 1H), 3.46-3.35 (m, 1H), 2.63-2.56 (m, 3H), 2.51-2.44 (m, 1H), 2.35-2.25 (m, 1H).

– characterization conformed to reported values.

Sonogashira coupling of DABCYL alkyne 1 to 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-5-iodouridine (V-2a)

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-5-iodouridine (0.500 g, 0.8 mmol), and DABCYL analog alkyne I (0.299 g, 1.2 mmol) were dissolved in DMF (7 ml) and triethylamine (0.162 g, 1.6 mmol) was added. The solution was cooled to -78°C and degassed. Pd(PPh₃)₄ (0.092 g, 0.08 mmol) and CuI (0.031 g, 0.16 mmol) were added under N₂ and the solution was stirred overnight in the dark at RT under a nitrogen atmosphere. The reaction mixture was partitioned in DCM/saturated EDTA and the organic phase was washed with sat. EDTA (3 x 25 ml). The organic phase was dried with Na₂SO₄ and solvent was removed. The crude mixture was purified by FCC eluting with 80:15:5 DCM:acetone:Et₃N to yield compound V-2a as a bright orange foam (0.394 g, 63%). 1H NMR (400 MHz, CDCl₃) δ: 8.23 (s, 1H) 7.86 (d, J = 8.99 Hz, 2H) 7.49-7.25 (m, 11H) 7.10-7.20 (m, 3H) 6.71-6.90 (m, 7H) 6.36 (s, 1H) 4.62 (m, 1H) 4.15
(m, 1H) 3.8 (m, 1H) 3.70 (d, J = 4.7 Hz, 6H) 3.60 (m, 2H) 3.46 (m, 1H) 3.37 (m, 3H) 3.10 (s, 6H) 2.54-2.64 (m, 2H) 2.37 (m, 1H).

**Cyclization to furanouridine (V-2)**

Compound **1-2a** (0.278 g, 0.4 mmol) was dissolved in DMF (4 ml), Et₃N (3 ml), and MeOH (6 ml). The solution was cooled to -78°C and degassed. CuI (0.0270 g, 0.14 mmol) was added and the solution was degassed again. The mixture was heated to 50°C and stirred in the dark under a nitrogen atmosphere for 72 hrs. Et₃N (3 ml) and CuI (0.0270 g, 0.14 mmol) were added and the mixture was stirred overnight @ 50°C in the dark under N₂. The mixture was partitioned in 50 ml DCM/saturated EDTA and washed with sat. EDTA (3 x 50 ml) and brine (1 x 50 ml). The organic phase was dried with Na₂SO₄ and solvent was removed. The mixture was purified by FCC eluting with 8.5:1.5:1:0.5 DCM/acetone/hexanes/Et₃N to yield an orange solid (0.1209 g, 44%). **¹H NMR (400 MHz, CDCl₃) δ:** 9.00 (s, 1H) 7.89 (m, 4H) 7.73 (m, 2H) 7.40-7.26 (m, 11H) 6.86 (m, 4H) 6.78 (m, 2) 6.35 (m, 1H) 5.72 (s, 1H) 4.75 (m, 1H) 4.15 (m, 1H) 3.76 (s, 6H) 3.61 (m, 3H) 3.11 (m, 5H) 2.76 (m, 1H) 2.50 (m, 1H).

**Aminolysis of compound V-2**

Compound **V-2** was placed in a pressure vessel and dissolved in 2-3 ml concentrated liquid ammonia. The dark brown mixture was stirred at room temperature overnight. NH₃ was removed by evaporation at room temperature/atmospheric pressure and the orange solid in the pressure vessel was dissolved in minimal DCM and precipitated in hexanes. The solid was collected by gravity filtration and solvent was removed under vacuum. The residue was purified by FCC (70:20:5:5 DCM/acetone/MeOH/TEA) and the fractions of interest were collected. HRMS (ESI): calculated for C₄₆H₄₆N₆O₇: 795.3744, found: 795.3729.

**Sonogashira coupling of DABCYL analog alkyne 1 to protected iodocytidine II-3 (compound V-6a)**

Compound **V-5** (0.700 g, 1.0 mmol), DABCYL analog alkyne **I** (0.3812 g, 1.5 mmol), Pd(PPh₃)₄ (0.1156 g, 0.1 mmol), and CuI (0.0190 g, 0.1 mmol) were dissolved in 6 ml fresh THF. Et₃N (3 ml) was added and the mixture was brought to -78°C and degassed. The mixture was heated to 55°C and stirred in the dark under N₂ for 48 hrs. Partitioned mixture in
DCM/saturated EDTA and washed with sat. EDTA (3 x 50 ml). The organic layer was dried with Na₂SO₄ and solvent was removed under vacuum. The residue was subjected to FCC (eluent gradient DCM - DCM:MeOH 100-95:5) and solvent was removed from fractions containing compound of interest which were then carried forward to benzoyl deprotection.

**Benzoyl Deprotection of Compound V-6a (Compound V-6)**

The residue from the previous reaction was suspended in a mixture of 2:1 anhydrous EtOH:Et₃N (30 ml). The suspension was heated to 55°C and stirred under N₂ overnight. The reaction was stopped and solvent was removed from the mixture under vacuum. The residue was subjected to FCC and fractions of interest were isolated and carried forward to the next step. ¹H NMR (400 MHz, CDCl₃) δ: 8.73 (s, 1H) 7.86-7.95 (m, 8H) 6.79 (m, 2H) 6.54 (s, 1H) 6.45 (m, 1H), 4.43 (m, 1H) 4.09-4.02 (m, 2H) 3.87-3.85 (m, 1H) 3.12 (m, 9H) 1.00 (s, 9H) 0.90 (s, 10H) 0.08 (m, 6H).

**TBDMS Deprotection of Compound V-6 (Compound V)**

Compound V-6 (0.4226 g, 0.6 mmol) was dissolved in 7 ml THF. The solution was cooled to 0°C and Et₃N • 3HF (0.2910 g, 1.8 mmol) was added dropwise. The mixture was brought to RT and stirred overnight. Solvent was removed and the residue was purified on a short silica gel column (90:10 DCM:MeOH) to yield compound V as an orange solid (0.2297 g, 81%). ¹H NMR (400 MHz, DMSO-d₆) δ: 11.89 (br. s., 1H) 8.76 (s, 1H) 7.97 (d, J = 8.6 Hz, 2H) 7.82 (m, 4H) 6.86 (d, J=9.4 Hz, 2H) 6.26 (m, 1H) 5.17 (s, 1H) 4.26 (m, 1H) 3.92 (m, 1H) 3.65 (m, 2H) 3.07 (s, 6H) 2.38 (m, 1J) 2.04 (m, 1H). ¹³C NMR (DMSO-d₆) 160.02, 153.80, 152.55, 151.84, 142.70, 138.64, 136.74, 131.36, 125.78, 124.85, 122.35, 111.56, 109.17, 98.16, 87.93, 87.08, 69.85, 60.93, 45.66, 41.49.

**Tritylation of Compound V (Compound V-3)**

Compound V (0.1991 g, 0.4 mmol) was suspended in anhydrous pyridine (15 ml) and dry Et₃N (2.5 ml). The suspension was cooled to 0°C. 4, 4’ dimethoxytrityl chloride (0.1849 g, 0.5 mmol) was dissolved in 3 ml anhydrous pyridine and this solution was cooled to 0°C, then added to the previous mixture dropwise via syringe. The mixture was brought slowly to RT and allowed to stir under N₂ for three days. The reaction mixture was partitioned in DCM/saturated NaHCO₃...
and washed with sat. NaHCO₃ (2 x 10 ml) and brine (1 x 10 ml). The organic phase was dried over Na₂SO₄, then concentrated and the residue was purified on a short silica gel column (95:5 DCM:MeOH) to yield an orange solid (0.1509 g, 49%). ¹H NMR (DMSO-δ₆): δ: 11.39 (s, 1H) 7.78 (d, J = 8.99 Hz, 2H) 7.41 (d, J = 8.21 Hz, 2H) 7.20-6.96 (m, 7H) 6.96 (m, 4H) 6.82 (m, 2H) 6.09 (m, 1H) 5.24 (m, 1H) 4.66 (m, 1H) 3.91 (m, 1H) 3.69 (m, 1H) 3.59 (s, 6H) 3.06 (m, 9H) 2.20 (m, 1H) ³¹C NMR (DMSO-δ₆) 157.96, 157.47, 153.55, 152.74, 151.13, 145.69, 142.81, 137.46, 137.21, 134.15, 131.58, 130.50, 130.14, 127.51, 124.94, 120.79, 117.28, 112.76, 111.76, 110.04, 87.77, 86.57, 70.92, 62.09, 58.21, 55.01, 40.35, 40.14. HRMS (ESI): calculated for C₄₆H₄₅N₆O₆: 777.3401, found: 777.3431.

**Phosphitylation of Compound V-3 (Compound V-34)**

Compound V-3 (0.1000 g, 0.1 mmol) was dissolved in dry DCM (2.5 ml) and dry DIPEA (0.0259 g, 0.2 mmol) was added via syringe. 2-cyanoethylisopropylphosphoramidite (0.0473 g, 0.2 mmol) was added dropwise via syringe and the reaction mixture was stirred under N₂ at RT until TLC indicated consumption of starting material (3.5 hrs). The reaction mixture was quenched with 0.2 ml MeOH, partitioned in DCM and saturated NaHCO₃, and washed with 5 ml saturated NaHCO₃ and 5 ml brine. The organic phase was dried over Na₂SO₄, then concentrated. The residue was dissolved in minimal DCM and precipitated from cold hexanes to yield an orange solid (0.0951 g, 76%). ¹H NMR (DMSO-δ₆) 11.46 (br s, 1H) 7.79 (m, 2H) 7.40 (m, 2H) 7.18-6.95 (m, 11H) 6.82 (m, 2H) 6.61 (m, 4H) 6.06 (m, 1H) 4.38-4.17 (m, 2H) 3.96-3.78 (m, 2H) 3.59 (s, 6H) 3.06 (s, 6H) 2.95 (m, 1H) 2.72 (m, 1H).
Chapter 4 – Conclusion and Outlook

This thesis reports progress toward the synthesis of PNA and DNA molecular beacons, as well as fluorophores and quenchers suitable for incorporation into molecular beacons. The first portion of the work centered on the development of a novel stepwise synthetic methodology that would enable the synthesis of a PNA molecular beacon differentially labeled with a fluorophore and a quencher by CuAAC. This synthesis would capitalize on two assets previously developed in the Hudson group: the DABCYL analog alkyne, and an Fmoc PNA monomer containing an azide group. Three fluorescent molecules were also derivatized with alkyne “handles” for click chemistry via simple, efficient syntheses: 9-aminoacridine, NBD, and acridone. While 9-propargylaminoacridine gave disappointing results in terms of fluorescence quantum yield, both the NBD and acridone alkynes were demonstrably viable fluorophores. Furthermore, a procedure for on-resin click chemistry was evaluated and found to be superior to conditions previously employed in the group in terms of efficiency of both reaction time and materials used. Unfortunately, technical issues related to PNA peptide synthesis deterred progress toward completion of the full molecular beacon; however, the groundwork has been laid to proceed with the synthesis once these difficulties have been overcome. Synthesis and evaluation of the differentially labeled molecular beacon would represent a significant contribution toward the development of a relatively fast and simple method for generating molecular beacons which could be used in the detection of genetic mutations. In addition, the “clickable” fluorophores themselves could be used for other applications, such as the labeling of other biomolecules, notably proteins.

The second part of the work focuses on the synthesis of a pyrroloctidine phosphoramidite containing a diarylazo moiety that is also analogous to the alkyne quencher DABCYL. The free nucleoside had previously been synthesized and its quenching properties determined, with encouraging results. Synthetic methodologies for the synthesis of the DABCYL pC phosphoramidite were pursued along two different pathways, with uridine and cytidine as departure points. The uridine pathway seemed promising, but stalled at the aminolysis step. The cytidine pathway enabled arrival at the free nucleoside, but routine protecting group chemistry employed in the conversion of the nucleoside to a phosphoramidite resulted in the apparent addition of the dimethoxytrityl group to the pC heterocycle by an unanticipated electrophilic
aromatic substitution. While the result was not desirable and prevented the incorporation of the DABCYL pC nucleoside into a DNA molecular beacon, this side reaction appears to be unprecedented and appears to affect a subset of electron-rich pyrrolocytidines that have been synthesized in the Hudson group. Thus, the result is intriguing and merits further study. Meanwhile, the optimization of synthetic steps along both the uridine and cytidine pathways may be valuable in future endeavours to complete synthesis of the DABCYL pC phosphoramidite and incorporate it into DNA. Further efforts will likely involve the development of alternative protecting group strategies for the cytidine pathway, or optimization of the aminolysis step of the uridine pathway.

In conclusion, this work represents progress toward novel molecular beacon synthetic strategies that may in future improve the ease of synthesis of these valuable molecular diagnostic tools.
References

2. Reid, J. B.; Ross, J. J. Genetics 2011, 189, 3-10.


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Curriculum Vitae

<table>
<thead>
<tr>
<th>Name:</th>
<th>Christie Ettles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post-secondary Education and Degrees:</strong></td>
<td></td>
</tr>
</tbody>
</table>
| University of New Brunswick  
Fredericton, New Brunswick, Canada  
2007-2011 B.Sc. |  |
| The University of Western Ontario  
London, Ontario, Canada  
2011-2013 M.A. (in progress) |  |
| **Honours and Awards:** | Western Graduate Research Scholarship  
Winter 2012, Fall 2012 |  |
| **Related Work Experience:** | Teaching Assistant  
The University of Western Ontario  
2012-2013 |  |
| | Graduate Research Assistant  
Department of Chemistry  
The University of Western Ontario  
2011-2013 |  |
| | Undergraduate Research Assistant  
Department of Chemistry  
The University of New Brunswick  
Summer 2011 |  |
| | Peer Assisted Learning Session Leader  
The University of New Brunswick  
2009-2011 |  |
| **Publications and Presentations:** |  |
| August 2012  
IS3NA International Roundtable on Nucleosides, Nucleotides and Nucleic Acids  
Montréal, Quebec  
Poster: Christie Ettles and Robert H. E. Hudson, “Multiple Labelling of PNA Oligomers by Sequential Azide-Alkyne Cycloaddition Reactions” |  |