Synthesis and Characterization of New Near-Infrared Chromophores: Cyanine and Phenoxazine Derivatives

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SYNTHESIS AND CHARACTERIZATION OF NEW NEAR-INFRARED CHROMOPHORES:
CYANINE AND PHENOXAZINE DERIVATIVES

by

EDUARDO SALVADOR SORIANO JUAREZ

Under the Direction of Professor Maged Henary, Ph.D

ABSTRACT

This thesis reports the synthesis of new near infrared dyes in three chapters. The first two chapters outline the synthetic procedure for synthesizing mono- and pentamethine cyanine dyes. The initial chapter encompasses the synthesis of asymmetric monomethine dyes with redshifted optical properties. The second chapter involves the synthesis and assessment of new symmetrical quinolin-4-yl and phenanthridin-6-yl pentamethine dyes as potential oxidative DNA cleavage agents. The last chapter of the thesis details the synthesis and evaluation of new phenoxizinum dyes as contrast agents for insulunomia, a pancreatic cancer. Furthermore, all new compounds were characterized via NMR and their coherent optical properties were obtained.

INDEX WORDS: cyanine dyes; phenoxazine; PDT agents; NIR Imaging; DNA cleavage
SYNTHESIS AND CHARACTERIZATION OF NEW NEAR-INFRARED CHROMOPHORE: CYANINE AND PHENOXAZINE DERIVATIVES

by

EDUARDO SALVADOR SORIANO JUAREZ

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2015
DEDICATION

I would like to dedicate this achievement to my loving parents, siblings and to all my family and friends.
ACKNOWLEDGEMENTS

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1 Cyanine Dyes

The proceeding chapter will focus on exploring the modifications to the general scaffold and the corresponding changes in optical properties and/or biological activity.

1.1 Introduction to Cyanine Dyes

Polymethine dyes and their derivatives such as carbocyanines, represent a class of organic molecules with absorption bands that covers a broad spectral range (400-1300 nm); larger than any other class of dye system\(^1,2\). A carbocyanine dye consists of two terminal aza-heterocycles connected via an electron deficient polymethine chain, as shown in Figure 1.

![Figure 1 Generic structure of cyanine dyes, polymethine core highlighted in red.](image)

The common name of cyanine dyes bearing the general structure, are derived by the number of n groups of the methine chain. For illustration, if n = 1, 2, 3, then the common names would be tri-, penta- and heptamethine cyanines, respectively, which indicate the number of methine unit (=C-H) between the aza-heterocycles. Due to the diversity in function associated with this class of chromophore, an extensive number of cyanine dyes have been synthesized for numerous applications in photographic processes and more recently as fluorescent probes for biomolecular labeling and imaging\(^2,4\). The applications of these dyes arise from the electron deficient \(\pi\) -conjugated system between the heteroatom that allow for cyanine dyes to achieve model photo-physical properties such as good molar absorption coefficients (>\(10^5\) M\(^{-1}\) cm\(^{-1}\)), tunable fluorescence intensities, and narrow absorption bands. One may modify the absorption band of cyanine dye by altering the conjugated system via the length of the polymethine chain, varying the heterocyclic ring(s), and substituents on either the heteroatoms and/or ring. As
many compositions of dyes can be made, it is noteworthy that to make cyanine dyes be both non-toxic and stable towards chemical decomposition based on the applications of the dye. For example, polar groups are added, such as sulfonates or carboxylates as substituents on the dye system to achieve to biocompatibility, as seen in the only FDA approved cyanine dye indocyanine green (ICG) in Figure 1.

![Figure 1 FDA approved cyanine dye, Indocyanine green (ICG)](image_url)

To the same degree, specific structural modifications allow for cyanine dyes to assume properties that would enable it to target selectively and maintain the selected fluorescence and/or absorption bands. This chapter will discuss the cyanine chromophore syntheses and relevance in future applications.

1.2 Synthesis of Monomethine Cyanine Dye

Monomethine cyanine dyes, in which there is only one methine unit (–CH–) between the two terminal heterocycles, commonly absorb between 480-515 nm. In general, monomethines are prepared through the condensation of two heterocyclic quaternary salts with a basic reagent. The requisites for the reactants includes: 1) one contain an active methyl group (promptly activated under the basic conditions) and 2) the other must possess a moiety that promotes the condensation reaction through an electrophilic site (electrophilic carbon attached to a good leaving group), as shown in Equation 1. Common moieties include: thioether, imino, sulfobetaines, halide, ketone or more recently, a thioketone moiety, as shown in Scheme 1.
Equation 1 General synthesis of asymmetric and symmetric monomethine dyes; Y = Anion; het = aza-heterocycle; LG = Leaving Group = -Cl, -SCH3, or -SO3-

Scheme 1 Synthesis of asymmetric monomethine cyanine via utilizing ketones and thioketone intermediate

A quaternary salt with an active methyl, such as N-methyl-2-methylbenzothiazole salt 1, can be condensed with a thione or ketone in basic solutions to afford asymmetric monomethine dyes 2 and 3, as shown in Scheme 1. In a similar manner, symmetrical monomethine dyes can be afford by utilizing either 3-methyl-2-iminobenzothiazolines or 3-methylbenzothiazoles-2-sulfonate salts, as other salt for the preparation of the dye 4 with salt 1, as shown in Scheme 16,7,10. Thus, monomethine dyes can be synthesized in both symmetrical and asymmetrical manners. Two asymmetrical dyes shown in Figure 2, Thaizole Orange (TO) and Oxazole Yellow (YO), are well known imagining probes in the biological sciences due to their enhanced photophysical properties upon binding to target macromolecule (i.e.nucleic acid structure protein, .etc.). These compounds, TO and YO can be prepared via a thioether pathway as shown in Equation 211-13.

Figure 2 Structures of Thaizole Orange (TO) and Oxazole Yellow (YO); X = Anion
The second chapter will utilize the thioether method for the synthesis of red-shifted monomethine dyes.

1.3 Synthesis of Polymethine Dyes

The synthetic preparation of polymethine dyes can be classified based on the intermediate used for the condensation. For example, trimethine dyes can be synthesized via three different methods, yielding symmetrical or asymmetrical trimethine dyes. The classical ortho ester method, established by Koenig et al., involves condensation of a quaternary salt with triethyl orthoformate under basic conditions in acetic anhydride to afford symmetrical trimethine 8, as shown in Equation 3.

Equation 3 condensation of a methyl-heterocycle with triethyl orthoformate in acetic anhydride to afford symmetrical trimethine

Applying this method, our lab has synthesized a series of halogenated trimethine dyes as potential probes for G-quadruplex detection in vivo. The dye system can also be modified to improve photostability and spectral properties, such as the introduction of a CN group onto the trimethine chain of cyanine dye 12 via an aldehyde pathway, as shown in Equation 4.
Equation 4 Asymmetrical trimethine dyes via an aldehyde pathway\textsuperscript{16}.

Trimethine dyes can also be synthesized in either both symmetrical and asymmetrical via the aldehyde pathway as shown in Scheme 2\textsuperscript{17}.

Scheme 2 Synthesis of trimethine via aldehyde method

This aldehyde method can also be applied to yield asymmetrical pentamethine dyes as shown in Scheme 3\textsuperscript{18}.

Scheme 3 Asymmetrical pentamethine dyes via an aldehyde intermediate

Lastly, tri-, penta-, and heptamethine cyanine syntheses can be achieved via the condensation reaction between a quaternary salt and methine-chain precursor 16, as shown in Scheme 4\textsuperscript{18-20}.
This method can be applied to synthesize either symmetrical 17 or unsymmetrical 19 dyes by controlling the ratio of the indole salt to polymethine chain linker 16, (N-diphenylformamidine, malonaldehyde dianil hydrochloride or glutacondianil hydrochloride for tri-, penta-, and heptamethine carbocyanines respectively). For unsymmetrical carbocyanine dyes, the reaction is proceeded with a quaternary ammonium salt with linker in a 1:1 ratio, yielding the hemicyanine 18, which is isolated and then reacted with another quaternary salt to afford dye 19. Symmetrical cyanine dyes, the reaction of quaternary ammonium salt with linker at a 2:1 ratio is carried out. Employing the hemicyanine method, the Henary group has synthesized sets of symmetrical pentamthine dyes compounds as potent and selective PRMT inhibitors and oxidatively cleave U19 plasmid DNA. Furthermore, our lab has also applied this method to afford penta- and heptamethine as novel NIR contrast agents, which were based on physicochemical properties to prompt tissue-specific uptake into sensitive tissues (i.e., endocrine glands).

1.4 Functionalization of cyanine dyes

Functionalization of cyanine dyes if needed, can be achieved in both asymmetrical and symmetrical dyes. As such conjugation of cyanine dyes includes the use of either a symmetrical or an unsymmetrical cyanine dyes with functional groups are on the polymethine chain or part of the terminal heterocycles as shown in Figure 3.
For example, magnetic nanoparticles (MNPs) utilize functionalized dyes with iron oxide (Fe$_3$O$_4$) as bioimaging agents that allow for direct labeling of cancerous HeLa cells via both optically (NIR fluorescence microscopy) and thru magnetic resonance (MR) imaging$^{31}$. The dye system can also be functionalized via encapsulation/micelle (micro- and nano-), as shown by ICG for self-assembly synthesis, tumor cell targeting, and photothermal capabilities$^{32-34}$. Furthermore, dyes can be conjugated as fluorescent labels to provide alternative methods to monitor intracellular reactions, such as the intracellular delivery of antisense peptide nucleic acid by fluorescent mesoporous silica nanoparticles$^{30}$. The diversity of the dye scaffold can be seen in the different substitution on the meso-carbon in both penta-and heptamethine. In the case of pentamethine dyes, our lab has shown bio-conjugation via the meso-carbon on the cyanine dye as methods for trafficking scaffold degradation$^{35}$. More recently, substitution at the center carbon with hydroxyethyl piperazine functionalized at the meso-carbon, allows for selective colorimetric and ratiometric absorption responses to trivalent metal cations$^{28}$.

2 Synthesis of Asymmetric Monomethine Cyanine Dyes with Red-Shifted Optical Properties.

*This current chapter will briefly report on the synthesis of asymmetric monomethine cyanine dyes with red-shifted optical properties. Additional modeling studies were conducted to further understand the dyes’ photo physical properties to determine how substitution affect the optical properties of the dyes. Part of this chapter of thesis was published in Journal of Heterocyclic Chemistry, (Vol 52, 180, 2015), and is presented thereafter in its published form*
Synthesis of Asymmetric Monomethine Cyanine Dyes with Red-Shifted Optical Properties

Eduardo Soriano, Loretta Butler, Eric A. Owens, and Maged Henary

Department of Chemistry and Biochemistry, Georgia State University, 100 Piedmont Avenue, Atlanta, Georgia 30303, USA

*E-mail: mcenary1@gsu.edu

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Six novel asymmetrical monomethine cyanine dyes were synthesized via the condensation reaction of 1-butyldimethylxilo[1:2]indol-1-ium iodide and various 1,5-substituted indolenine salts under basic conditions. The dyes were characterized using UV–vis spectroscopy, fluorescence, 1H NMR, 13C NMR, and mass spectrometry; furthermore, the purity of these compounds was observed using LC/ELSD/MS.


INTRODUCTION

The first cyanine dye synthesized was derived from the heating of N-vinyl quinolinium iodide with N-vinyl lepidinium iodide in ammonia, forming a blue compound. Since this synthesis in 1856 by C. G. Williams, cyanine dyes have been extensively employed in various applications such as photographic processes, laser printing, nonlinear optical material, and more recently as fluorescent probes for biomolecular labeling and imaging [1–6]. In general, these dyes consist of two terminal heterocyclic rings with nitrogen centers that are connected via a conjugated and electron-deficient methine chain. Common heterocyclic moieties include quinoline, indolenine, and benzothiazole. (Figure 1)

The applications of these dyes arise from the electron-deficient conjugated system present between the nitrogen centers. With current synthetic methods, the conjugated system of the compounds can be altered to assume specific absorption and fluorescence spectra within the range of 400 to 1000 nm [7,8]. The dyes presented in this paper are monomethine cyanine dyes, in which there is only one carbon atom between the two terminal heterocycles that commonly absorb between 480 and 515 nm [7,8].

Monomethine cyanine dyes generally have high molar extinction coefficient values and exhibit little to no fluorescence as a free dye in solution unless conformationally restricted [9,10]. These properties provide ideal fluorogenic characteristics that allow them to be among the best noncovalently binding nucleic acid labels [9,10]. Additionally, when these dyes are within a viscous environment, their fluorescence becomes observable, which allows for viscosity-dependent fluorescence measurements and direct analyses of various environments in biological systems [9]. These applications prompted the synthesis of 1,5-substituted monomethine cyanines that have large Stokes shift values and red-shifted absorbance and fluorescence properties.

Synthesis and photophysical determinations. The synthetic preparation of asymmetric monomethine cyanines begins with the formation of the indole salt (as shown in Scheme 1); the reaction between the 4-substituted phenylhydrazines 1–3 with the 3-methylbutan-2-one (4) results in an enamine. Upon the formation of the enamine, the acidic environment catalyzes the pericyclic rearrangement of the enamine that is energetically driven by the formation of the cyclic indole. A saturated solution of sodium bicarbonate was used to neutralize the reaction mixture due to the increase of ammonium as byproduct from the cyclization and to ensure the deprotonation of the indolenine nitrogen for subsequent reactions.

After forming the 5-substituted indolenine ring 5–8, various alkyl halides were used for quaternizing the N-indolenyl moiety. The alternate half of the monomethine dye was then prepared starting from the commercially available anide 15. This reagent was alkylated using iodotoluene, and the carbonyl substituent was then transformed to the thione using phosphorous pentasulfide. This was then transformed to the methylsulfide using iodomethane for the reaction with the previously prepared indolenine salt. The synthesis of the monomethine cyanine dyes was then achieved via the condensation reaction of 1-butyldimethylxilo[1:2]indol-1-ium iodide (18) and an indolenine salt in the presence of the basic agent, TEA. The base within the reaction was needed for the activation
of indolium salt via the deprotonation of the methyl at the 2 position. After the deprotonation, the mechanism proceeds in an $S_2$ fashion in which the activated methylene group of the various individual salts 9–14 displaces the methyl sulfide moiety of 18 and results in the formation of the asymmetrical monomethine dyes 19–24.

Both UV–vis and emission spectra were recorded for each dye in MeOH and 90% glycerol/10% MeOH, shown in Table 1. There are two molecular attributes that account for the red-shifted absorbance wavelengths of the presented dyes. The electron-deficient conjugated system is responsible for the red-shifted absorbance wavelengths when compared with a polynene system; additionally, the benzene($c,d$) indole moiety provides increased conjugation within the chromophore and an additional red shift of approximately 50 nm in absorbance when compared with previously synthesized asymmetric monomethine cyanines [1–3].

There was an average of 7 nm bathochromic shift in absorbance maxima in the 90% glycerol/10% MeOH (v/v) solution from the newly synthesized dyes. Furthermore, there was a decrease in the molar absorptivity values for a majority of the dyes synthesized. Methanol was used to solubilize the compounds in the highly viscous glycerol. A common characteristic observed with this set of dyes is their broad absorbance spectra that can be attributed to a high level of aggregation, as shown using compound 23 in Figure 2 [1].

Furthermore there was an average of 88 nm Stokes shift for each of the dyes in the same solution. Large Stokes shift is important for it increases the utility of the fluorophore as the excitation source remains far from the fluorescence signal, which reduces Rayleigh scattering, and such properties are useful for in vitro and in vivo imaging especially in highly scattering media [13,14]. The fluorescence of these compounds could not be observed in methanol alone because of the free rotation around the internuclear bridge resulting in a high nonradiative rate of return of the excited molecule—as cited with many monomethine cyanines containing the indolenine structure [4,11,12]. In viscous solution of 90%

---

**Table 1**

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{max}$ (nm)$^a$</th>
<th>$\lambda_{sh}$ (nm)$^b$</th>
<th>$\lambda_{em}$ (nm)$^b$</th>
<th>Stokes shift (nm)$^b$</th>
<th>( \varepsilon ) (L mol(^{-1}) cm(^{-1})) (^c)</th>
<th>( \varepsilon ) (L mol(^{-1}) cm(^{-1})) (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>556</td>
<td>565</td>
<td>657</td>
<td>92</td>
<td>30,000</td>
<td>26,200</td>
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<td>658</td>
<td>89</td>
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<td>27,300</td>
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<td>575</td>
<td>662</td>
<td>87</td>
<td>33,500</td>
<td>39,900</td>
</tr>
</tbody>
</table>

\(^a\)in methanol.
\(^b\)in 90% glycerol/10% methanol (v/v).

---

Journal of Heterocyclic Chemistry  DOI 10.1002/jhet
glycerol/10% MeOH (v/v), a fluorescence signal was observed because of the hindered rotation of the dye molecule.

CONCLUSIONS

A series of six monomethine derivatives were successfully synthesized in good yield with large Stokes shifts and red shifted absorbance and fluorescence properties in comparison with previously synthesized asymmetric monomethines. The dyes' observable fluorescence in a highly viscous environment such as the glycerol–methanol solution reveals for its use to report on viscosity, which could be beneficial for evaluating nonsolvent interactions that restrict the rotation of the dye. Incorporating the benzofuran indole moiety afforded the red-shifted optical properties desired. Lastly, the synthetic chosen synthetic route requires a facile purification methodology, which allows for the further development of this class.

MATERIALS

Chemicals used: dichloromethane (DCM; reagent grade, >95.5% purity, Sigma Aldrich, St. Louis, MO), diethyl ether (reagent grade, >99% purity, Sigma Aldrich), acetic anhydride, sodium acetate (99.5% purity, Merck KGaA, Darmstadt, Germany), isopropanol (99% purity, Sigma Aldrich), 2,3,3-trimethylindoline (99% purity, Sigma Aldrich), methanol (reagent grade, >99.5% purity, Sigma Aldrich), hexanes (reagent grade, >95.5% purity, Sigma Aldrich), 4-(bromophenyl)hydrazine hydrochloride (99%, Acros Organic, Belgium, Germany), 3-methylindolin-2-one (≥99% purity, Sigma Aldrich), TEA (99% purity, Fisher Chemical, Germany), isobutyl ether (99%, Alfa Aesar, Lancashire, UK), 3-bromo-1-phenylpropane (99%, Alfa Aesar), glycerol (A.C.S. certified, 99.6% purity, Fischer Scientific Company, Fair Lawn, NJ).

Chromatography: pre-coated TLC plates, silica gel 60 F-254 (Merck KGaA). For Rf pre-coated TLC plates, Whatman Pasteur CS5 Multi K with fluorescent indicator, plate thickness 250 μm (Maidstone, Kent, UK). NMR data were obtained from a Bruker Avance 300 and 400 MHz NMR spectrometer (Bruker Biospin Corporation, Newark, DE). The mass spectra were obtained using a Waters Micromass LCT TOF ES+ Premier Mass Spectrometer. Liquid chromatography utilized a Waters 2487 single wavelength absorption detector with wavelengths set between 640 and 700 nm depending on the dye’s photophysical properties. The column used in LC was a Waters Delta-Pak 5 μm 100 A 3.9 mm × 150 mm reversed-phase C18 column. Evaporative light scattering detection analyzes trace impurities that cannot be observed by alternate methods; a SEDEX 75 ELSD was utilized in tandem with liquid chromatography to observe and confirm purity.

Optical measurements. Molar absorptivity. The absorption spectra were recorded on a Varian Cary 3G UV–Vis spectrophotometer interfaced with Cary WinUV Scan application v3.00 at 20 μM in cuvettes with a path length 1 cm. For each optical measurement, stock solutions were prepared by adding the appropriate mass of each individual compound on a four-digit analytical balance to make a 1.0 mM solution. From these stock solutions, the dilutions seen in Supplementary information were obtained to determine the molar absorptivity values in methanol. For molar absorptivity values in glycerol–methanol (90%/10% v/v), the concentrations for the solutions were prepared via the dilution of the stock solution in methanol and followed the addition of the appropriate volume of glycerol to achieve the desired concentrations. Furthermore, each solution was vortexed for 1 min and sonicated for 10 min, and its absorbance was measured.

Fluorescence data. Fluorescence spectra for all monomethine cyanine dyes were obtained using a Shimadzu RF-5301 spectrophotometer (Shimadzu Corporation Analytical Instruments Division, Duisburg, F. R. Germany). The parameters implemented for this procedure were as follows: slit width for both excitation and emission 5 nm, sensitivity was set to high, wavelength speed set to medium; excitation wavelength 550 nm, and the light source was a 150 W Xenon lamp. The fluorimeter was interfaced to a PC, and data were collected using RF-5301PC software. Disposable polystyrene fluorescence cuvettes with a 1.00 cm path length were used for all measurements. All fluorescence measurements were made using solutions of A < 0.1 to avoid inner filter effects. The data were analyzed, and calculations were carried out using Microsoft Excel (Microsoft Corporation, Redmond, WA).

EXPERIMENTAL

General synthetic procedure for the indolium salts. Compounds 9–13 were synthesized as previously reported [3]. Each compound was then dissolved in acetonitrile (25 μL) and 4 molar
equivalences of the primary alkyl halide were added to the solution. The reaction mixture was then refluxed at 90°C for 72h. TLC was used to monitor the reaction in a solution of 4:1, DCM:hexanes. Upon cooling, the reaction mixture was returned to room temperature and acetone was added followed by diethyl ether to precipitate the salt. The solid was filtered and washed with diethyl ether. The salts were washed without further purification in subsequent reactions.

**Synthesis of 5-bromo-2,3,3-dimethyl-1-(3-phenylpropyl)-3H-indole-1-tiam bromide (14)**. Compound 7 (0.02 mmol) was dissolved in acetonitrile (25 mL), and 1-bromo-3-phenylpropene (0.044 mmol, 20 mL) was added. The reaction mixture was then refluxed at 90°C for 72h. TLC was used to monitor the reaction using DCM:hexanes (4:1) as eluting solvent. The reaction mixture was then allowed to cool to room temperature, acetone was added to the mixture, followed by diethyl ether to precipitate the solid. The solid was collected via vacuum filtration and washed with diethyl ether to afford 14 as a dark brown solid. Yield 43%, 3.5 g, mp 169-161°C. 1H NMR (400 MHz, CDCl3): 1.60 (s, 6H), 2.29 (m, J=8.0 Hz, 2H), 2.88 (s, J=8.0 Hz, 2H), 3.03 (s, 3H), 4.79 (t, J=8.0 Hz, 2H), 7.29 (d, J=4.0 Hz, 2H), 7.60 (d, J=8.0 Hz, 1H), 7.64 (d, J=6.0 Hz, 2H); δ ppm 14.23, 25.79, 49.06, 54.83, 106.17, 112.47, 112.74, 124.90, 128.59, 128.98, 132.40, 139.57, 140.17, 143.62, 195.46.

**General synthesis of the monomethine dyes**. Compound 18 was dissolved in 10 mL of CH3CN, and 1.5 equiv each of the appropriate indolium salt and TEA was added to the solution. The mixture was refluxed at 60°C for 1h. UV–vis was used to monitor the reaction. Upon cooling to room temperature, diethyl ether was added to precipitate the dye. The solid was collected and washed with deoxygenated water and diethyl ether. The dyes were purified via recrystallization by solution in a minimal amount of methanol and dilution with diethyl ether causing precipitation as dark purple solids.

(E)-1-Butyl-2-((E)-1-buty1-5-bromo-3,3-dimethylindolin-2-ylidene) methylbenzyl[3]azafullerene-1-tiam iodide (19). Yield 66%, 0.48 g, mp 220-221°C. TLC (25:1 CH3Cl:CH2Cl2): Rf = 0.05. 1H NMR (400 MHz, DMSO-d6): δ ppm 0.63 (t, J=7.3 Hz, 3H), 0.92 (t, J=6.7 Hz, 3H), 1.02 (q, J=7.0 Hz, 2H), 1.41 (q, J=5.4 Hz, 2H), 1.65 (s, 8H), 1.82 (q, J=7.3 Hz, 2H), 4.19 (q, J=2.3 Hz, 4H), 4.49 (s, 2H), 6.31 (s, 1H), 7.42 (t, J=9.8 Hz, 1H), 7.55 (t, J=8.9 Hz, 1H), 7.73 (m, 4H), 7.91 (m, 2H), 3.01 (d, J=7.0 Hz, 3H), 8.36 (d, J=7.21 Hz, 2H). 13C NMR (100 MHz, DMSO-d6): δ ppm 12.54, 13.35, 18.34, 19.15, 23.57, 28.89, 29.53, 43.27, 49.78, 51.32, 83.57, 110.71, 113.99, 122.82, 129.95, 123.46, 126.95, 128.14, 128.79, 129.2, 132.41, 140.34, 140.55, 141.94, 155.54, 180.09, 198.95. MS: m/z 432.2375, found m/z 432.2447.

(E)-1-Butyl-2-((E)-1-buty1-5-bromo-3,3-dimethylindolin-2-ylidene) methylbenzyl[3]azafullerene-1-tiam iodide (20). Yield 34%, 0.4 g, mp 197-198°C. TLC (25:1 CH3Cl:CH2Cl2): Rf = 0.08. 1H NMR (400 MHz, DMSO-d6): δ ppm 0.63 (s, J=7.3 Hz, 3H), 0.92 (t, J=8.9 Hz, 3H), 1.01 (m, 3H), 1.41 (m, 2H), 1.59 (m, 2H), 1.64 (s, 6H), 1.81 (m, 2H), 4.14 (m, 2H), 4.52 (m, 2H), 6.39 (s, 1H), 7.62 (d, J=8.0 Hz, 1H), 7.72 (d, J=7.4 Hz, 1H), 7.78 (m, 2H), 7.90 (m, 3H), 8.09 (d, J=8.0 Hz, 1H), 8.39 (d, J=7.0 Hz, 1H). 13C NMR (100 MHz, DMSO-d6): δ ppm 13.26, 13.66, 18.46, 25.65, 29.03, 29.93, 43.75, 50.26, 51.58, 84.00, 111.73, 115.66, 123.55, 123.66, 128.57, 128.84, 129.00, 129.13, 129.27, 129.56, 129.98, 130.09, 150.62, 133.14, 140.52, 146.83, 142.59, 156.40, 179.89, mp 197-198°C. λmax 562 nm in MeOH. IRMS (ESI) Calcd. for [C38H32CINO4]8+ m/z 458.0568, found m/z 458.7525.

(E)-1-Butyl-2-((E)-1-buty1-5-bromo-3,3-dimethylindolin-2-ylidene)methylbenzyl[3]azafullerene-1-tiam iodide (21). Yield 59%, 0.24 g, mp 174-175°C. TLC (25:1 CH3Cl:CH2Cl2): Rf = 0.8. 1H NMR (400 MHz, DMSO-d6): δ ppm 0.93 (t, J=9.0 Hz, 3H), 1.39 (m, J=9.0 Hz, 2H), 2.25 (s, J=9.0 Hz, 3H), 1.77 (m, J=9.0 Hz, 2H), 4.35 (t, J=9.0 Hz, 2H), 7.46 (d, J=6.0 Hz, 1H), 7.61 (d, J=6.0 Hz, 1H), 7.77-7.84 (m, 2H), 8.15 (s, 1H), 8.64 (d, J=6.0 Hz, 1H). Synthesis of 1-butyl-2-(dimethylamino)benzyl[3]azafullerene-1-tiam iodide (16a). A mixture of compound 17 and iodomethane (14 equiv) in the absence of solvent was heated to reflux for 8h. The iodomethane was removed in vacuo. The resulting residue was treated with diethyl ether, and the mixture was sonicated for 30min and was suction filtered, washed with diethyl ether, and dried leaving an 82% yield. 1H NMR (400 MHz, CDCl3): δ ppm 0.62 (q, J=7.6 Hz, 3H), 1.39 (q, J=6.7 Hz, 3H), 1.82 (m, J=7.2 Hz, 2H), 4.51 (t, J=7.2 Hz, 2H), 7.72 (d, J=8.0 Hz, 1H), 7.99 (t, J=7.6 Hz, 1H), 8.10 (d, J=8.0 Hz, 1H), 8.21 (d, J=7.6 Hz, 1H), 8.50 (d, J=7.6 Hz, 1H), 8.59 (s, J=8.0 Hz, 1H). 13C NMR (100 MHz, CDCl3): δ ppm 12.73, 17.73, 19.16, 29.47, 47.41, 117.02, 121.76, 127.29, 129.97, 128.12, 128.83, 130.47, 135.05, 136.93, 137.32, 171.74.

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(E)-3-carboxy-3-chloro-3,3-dimethyl-1,3-dioxolan-2-ylidene)-methyl)-1-butylnitrile, 1-iodo-1-ium iodide (29). Yield 54%; 0.46 g, m.p. 157–160°C; TLC (25:1 CHCl₃-CH₂Cl₂-CH₃OH): Rf = 0.16; ¹H NMR (400 MHz, DMSO-d₆): 6 ppm 0.96 (t, J = 6.4 Hz, 3H), 0.89 (t, J = 6.4 Hz, 3H), 0.98 (m, J = 7.0 Hz, 2H), 1.39 (m, J = 6.4 Hz, 2H), 1.58 (t, J = 5.8 Hz, 2H), 1.67 (s, 6H), 1.82 (m, 2H), 4.13 (s, 2H), 4.54 (s, 2H), 6.34 (s, 1H), 7.67–7.77 (m, 4H), 7.90 (m, 2H), 8.02 (s, 1H), 8.06 (d, J = 6.7 Hz, 1H), 8.37 (d, J = 7.4 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): 6 ppm 12.78, 13.19, 18.19, 18.98, 25.57, 28.55, 29.47, 43.44, 48.09, 49.84, 51.14, 83.69, 111.33, 115.64, 118.25, 123.09, 123.34, 123.38, 128.58, 129.05, 129.43, 129.66, 130.69, 132.64, 139.95, 140.78, 142.41, 155.95, 179.08; λₑₓ = 565 nm in MeOH; HRMS (ESI) Calcd. for [C₉H₉BrN₂]⁺ m/z 502.5078, found m/z 502.5074.

(E)-3-carboxy-3-chloro-3,3-dimethyl-1,3-dioxolan-2-ylidene)-methyl)-1-butylnitrile, 1-iodo-1-ium iodide (29). Yield 54%; 0.46 g, m.p. 157–160°C; TLC (25:1 CHCl₃-CH₂Cl₂-CH₃OH): Rf = 0.16; ¹H NMR (400 MHz, DMSO-d₆): 6 ppm 0.96 (t, J = 6.4 Hz, 3H), 0.89 (t, J = 6.4 Hz, 3H), 0.98 (m, J = 7.0 Hz, 2H), 1.39 (m, J = 6.4 Hz, 2H), 1.58 (t, J = 5.8 Hz, 2H), 1.67 (s, 6H), 1.82 (m, 2H), 4.13 (s, 2H), 4.54 (s, 2H), 6.34 (s, 1H), 7.67–7.77 (m, 4H), 7.90 (m, 2H), 8.02 (s, 1H), 8.06 (d, J = 6.7 Hz, 1H), 8.37 (d, J = 7.4 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): 6 ppm 12.78, 13.19, 18.19, 18.98, 25.57, 28.55, 29.47, 43.44, 48.09, 49.84, 51.14, 83.69, 111.33, 115.64, 118.25, 123.09, 123.34, 123.38, 128.58, 129.05, 129.43, 129.66, 130.69, 132.64, 139.95, 140.78, 142.41, 155.95, 179.08; λₑₓ = 565 nm in MeOH; HRMS (ESI) Calcd. for [C₉H₉BrN₂]⁺ m/z 502.5078, found m/z 502.7264.

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REFERENCES AND NOTES

2.1 Rationale

When constructing new dyes for specific application, it is important to understand how varying the substituents on the dye would affect the optical properties of the dye. These changes can be made to improve solubility, increase binding and/or permeability, but these structural modifications also cause changes in both the absorption and fluorescence bands. Although many unsymmetrical monomethine dyes have been made, there is a lack of current models for monomethine dyes with absorbance and fluorescence bands at wavelengths longer than 600 nm. Additionally, the Stokes shifts of monomethine dyes are narrow (<50 nm). These limitations prompted the synthesis of new monomethine cyanine dyes that would have large Stokes shift values and red-shifted absorbance and fluorescence properties. Herein we report the synthesis of two series of unsymmetrical monomethine cyanine dyes with indolenine moieties to determine how substitutions affect the optical properties of the dyes. Furthermore, optical, NMR and modeling data were collected to assist in understanding how these substitution affect the optical properties of the dyes.

2.2 Synthesis of Red Shift Monomethine dyes

![Scheme 5 Synthesis of red-shifted monomethine dyes](Image)
The synthetic preparation of the first set of asymmetric monomethine dyes 27a-g begins from the commercially available amide 20, constituting half of the dye, as shown in Scheme 6. This amide was then alkylated using iodobutane. The alkylated product 21 was then later transformed into the thione 22 using phosphorous pentasulfide in pyridine at reflux. The thione was then converted into the methylsulfide 23 by means of iodomethane at reflux. The alternate half of the asymmetric dye begins with formation of the indole salt 25 (as shown in Scheme 6) via the alkylation the 5-substituted indoles 24 under neat conditions. The synthesis of the indole begins with the reaction between the 4-substituted phenylhydrazines 24a-d with 3-methylbutan-2-one that forms an enamine intermediate, which later undergoes a pericyclic rearrangement that is both catalyzed by the acidic environment and energetically driven by the formation into the aromatic indole 25. The workup of that reaction included the use of a saturated solution of sodium bicarbonate to neutralize the reaction mixture (due to the increase of ammonium as byproduct from the cyclization) and to ensure the deprotonation of the indolenine nitrogen for subsequent reactions. After isolating the 5-substituted indolenine ring 25, various alkyl halides were used for quaternizing the N-indolenyl moiety for the reaction with the prepared salt 23. The synthesis of the monomethine cyanine dyes was then achieved via the condensation reaction of 1-butyl-2-(methylthio)benzo[cd]indol-1-iium iodide 23 and an indolenine salts 26a-g in the presence of the basic reagent, triethylamine (TEA) in acetonitrile. The base was needed for the activation of indolium salt via the deprotonation of the methyl at the 2 position of the indole. After the deprotonation, the mechanism proceeds in a S$_\text{N}$_2 fashion in which the activated methylene group of the salts 25, displaces the methyl sulfide moiety of 23 and results in the formation of the asymmetrical monomethine dyes 27a-g. The dye reactions were monitored via both TLC and UV-Vis for completion. The dye reaction was completed when there was no change in the spectra of the reaction mixture and confirmed with TLC in a 1:20 MeOH:DCM (V/V) mixture. After completion, the reaction mixture was concentrated under reduced pressure and dissolved in DCM to filter off the conjugate acid of TEA (triethylammonium chloride).
Scheme 6 Synthesis of monomethine dyes

The preparation of second set of the monomethine dyes 30a-c, shown in scheme 7 begin with the methylation of the nitrogen of the commercially available reagents 28a-c, 2-methylbenzothiazole, 2-methylbenzoxazole or 1,1,2-trimethylbenzo[e]indole respectively. After alkylation using iodomethane, the heterocyclic salts 29a-c were condensed with salt (23) in the presence of the TEA in acetonitrile. The dye reactions were monitored via both TLC and UV-Vis for completion. The dye reaction was completed when there was no change in the spectra of the reaction mixture and confirmed with TLC in a 1:20 MeOH:DCM (V/V) mixture. Purification of the dyes was achieved via selective precipitation from MeOH in ether. After isolation, the dyes were characterized and their photo physical properties of the dyes were recorded.

2.2.1.1 Optical Measurements Experimental

2.2.1.1.1 Molar Absorptivity

The absorption spectra were recorded on a Varian Cary 3G UV-Visible Spectrophotometer interfaced with Cary WinUV Scan Application v3.00 at varying concentrations (µM) in cuvettes with a pathlength 1 cm.

For each optical measurement, stock solutions were prepared by adding the appropriate mass of each individual compound on a five digit analytical balance to make a 1.0 mM solution. From these stock solutions, the dilutions seen in appendix were obtained to determine the molar ab-
sorptivity values in methanol. For molar absorptivity values in glycerol/methanol (90% / 10% v/v), the concentrations for the solutions were prepared via the dilution of the stock solution in methanol and followed the addition of the appropriate volume of glycerol to achieve the desired concentrations. Furthermore, each solution was vortexed for 1 min and sonicated for 10 min and then its absorbance was measured.

2.2.1.1.2 Fluorescence Data

Fluorescence spectra for all monomethine cyanine dyes were obtained using a Shimadzu RF-5301 Spectrofluorophotometer (Shimadzu Corporation Analytical Instruments Division, Duisburg, F. R. Germany). The parameters implemented for this procedure were as follows: slit width for both excitation and emission 5 nm; sensitivity was set on high; wavelength speed set to medium; excitation wavelength 550 nm, and the light source was a 150 W Xenon lamp. The fluorimeter was interfaced to a PC and data was collected using RF-5301PC software. Disposable polystyrene fluorescence cuvettes with a 1.00 cm pathlength were used for all measurements. All fluorescence measurements were made using solutions of A < 0.1 to avoid inner filter effects. The data was analyzed and calculations were carried out using Microsoft Excel (Microsoft Corporation, Redmond, Wa).

2.2.1.2 Computational methods

The Calculated LUMO and HOMO orbitals were obtained using a restricted hybrid HF-DFT SCF calculation performed using Pulay DIIS + Geometric Direct Minimization and 6-31G* basis set, B3LYP Method via Spartan or Gassuioin(03).

2.3 Characterization

Compound 27b was chosen as a representative for complete chemical characterization and analysis. $^1$H NMR spectra of compound 27b was measured in DMSO-$d_6$, is shown in Figure 4.
The aromatic region from $^1$H NMR spectra of compound 27b measured in DMSO-d$_6$ can be seen in Figure 5.

Figure 4 $^1$H NMR spectrum of 27b in DMSO-d$_6$ at 25°C

Figure 5 Aromatic region of $^1$H NMR spectrum of 27b in DMSO-d$_6$ at 25°C

The structure of the dye can be classified into three parts and were colored accordingly, the two end terminals and the methine unit, as shown in Figure 5. As such, the structure of the dye would produce eleven signals in the aromatic region: four from the indole in red, one from the
proton of the methine and six from the benz[c,d]indole in blue. The aliphatic region of the spectra would have nine signals, one from the dimethyl’s of the indole in red and the butyl chains on the heteroatoms would provide eight. Analyzing the spectra in Figure 5, the proton from the methine bridge would produce a singlet and is account for at 6.32 ppm, labeled in green in the spectra. The indole in red would provide a pair of triplets and doublets in the aromatic region of the spectra. The peaks at 8.36 (d, $J = 7.85$ Hz) 8.01 (d, $J = 7.34$ Hz), 7.55 (t, $J = 7.77$ Hz), and 7.43 (t, $J = 7.34$ Hz) follow the expected splitting pattern from the red indole. Furthermore the integration, and the similar coupling constants ($7.34-7.85$ Hz), of these four peaks would suggest that the protons from the indole in red. The peak 7.89 (t, $J = 5.63$ Hz) has a smaller couplings value when compared to the other peaks; this suggest that it is in a different environment from the other peaks. The benz[c,d]indole would produce a splitting pattern of two triplets and four doublets. The multiplet at 7.70-7.77 integrates to four protons and the peak at 7.89 (t, $J = 5.63$ Hz) integrates to two proton; based on the integration of a total of six proton from these peak in blue would correspond to the benz[c,d]indole.
For compound 27b, there would a total of 29 distinct carbon on the molecule but only 27 signals from the carbons appear in the $^{13}$C NMR spectrum as seen in Figure 6. The $^{13}$C NMR spectrum of 27b measured in DMSO-$d_6$ shows all 10 aliphatic carbons below 60 ppm. In the aromatic region of the $^{13}$C spectra, 16 of the aromatic carbons from the heterocycles appear with the range of 110 – 180 ppm with the peak at 83.93 ppm would correspond to the carbon of the methine bridge due to the sp2 nature of the carbon. All carbons except for two are accounted for, in which it is assumed that two of the five quaternary aromatic carbons are missing and would need more scans to show up on the spectra. The positive charge of the dyes is delocalized as shown in Figure 7. The LUMO and HOMO orbitals for dye 27b in Figure 8

![Figure 6 $^{13}$C NMR spectrum of 27b measured in DMSO-$d_6$ at 25° C]

![Figure 7 Resonance structure]
Figure 8 Calculated LUMO (left) and HOMO (right) orbitals of 27b using Density Functional B3LYP force field and 6-31G* data set.

From the above diagram the majority HOMO orbitals are located are benz[c,d]indole moiety of the dye, demonstrating that the benz[c,d]indole acts as an electron sink, which account for the red-shift in absorbance bands due to further delocalization the positive charge on the dye molecule. In contrast the LUMO orbitals are spread throughout the dye molecule, the majority of the orbitals across the methine chain and the indole. This model suggests that the indole has a higher electron density as shown by the HOMO calculations. Furthermore this electron density was also reflected in the $^1$H spectra, which the peaks of the indole appear in an alternating manner. These peaks are shifted based on the alternating density of connected carbons.
Figure 9 ESI mass spectra of 27b
The mass spec was obtained via TOF ES+ wavelength absorption detector, ELSD, ESI mass spec and was utilized confirm purity, as show in Figure 9. The mass spectra showed only one ion peak that corresponds to the molecular weight of the compound that matches the calculated molecular weight of the dye 27b. All other spectra also showed a single signal that would correspond to the dye only bring present

2.4 Results and Discussion
UV-Vis and emission spectra were recorded for dyes 27a, 30a-c in both MeOH and 90% glycerol / 10% MeOH solutions as shown in Table 1. UV-Vis and emission spectra were recorded for dyes 27b-g in 90% glycerol / 10% MeOH solutions, as shown in Table 1.

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<th>Stokes Shift (nm)&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>72</td>
<td>38</td>
<td>25300</td>
<td>N.R.</td>
</tr>
</tbody>
</table>

Table 1 Wavelength of maximum for both absorbance and fluorescence and molar absorptivity in select solvent: <sup>a</sup> in methanol; <sup>b</sup> in 90% glycerol/10% methanol (v/v); <sup>c</sup> in methanol; N.O. Not observed in solution; N.R. Not recorded
When comparing dyes with and without substitutions at the 5-position of the indole, the compound fluorescence intensity decreases when compared to one another. The heavy atom effect has been shown to stabilize the excited state upon absorption of a photon which decreases limits the compounds ability to emit photons. In addition to this the fluorescence of these compounds could not be observed in methanol alone because of the free rotation around the internuclear bridge resulting in a high nonradiative rate of return of the excited molecule—as cited with many monomethine cyanines containing the indolenine structure.\textsuperscript{38-40}

However, in viscous solution of 90% glycerol/10% MeOH (v/v), a fluorescence signal was observed because of the hindered rotation of the dye molecule. Methanol was used in order to solubilize the compounds in the highly viscous glycerol. There was an average of 7 nm bathochromic shift in absorbance maxima in the 90% Glycerol / 10% MeOH (%v/%v) solution from the newly synthesized dyes 27b-g. Furthermore there was a decrease in the molar absorptivity values for a majority of the dyes synthesized. The electron deficient and conjugated system account for the red shifted absorbance wavelengths of the presented dyes would be responsible for the red shifted absorbance wavelengths when compared to a polyene system. the benzo[c,d]indole moiety provides increased conjugation within the chromophore and an additional red shift of approximately 50 nm in absorbance when compared with previously synthesized asymmetric monomethine cyanines.\textsuperscript{2,41,42} The substitution on the 5 position of the indole by an electron withdrawing or electron donating group resulted in a red-shift of absorbance maxima via inductive effect of the electron system as shown in Figure 10 by the electronic potential maps (EMP) of dyes 27b-e.
The inductive effect of different substituent is greater pronounced when comparing the molar absorptivity ($\varepsilon$) of the dyes. In addition, the computed charges for methine carbon following the same trend, as shown in Table 2. An electron donating group decreased the $\varepsilon$ value, due from increasing electron density thru the methoxy substituent. In contrast, the indoles with the electron withdrawing halogens increased the $\varepsilon$ value for the system when both are compared to the non-substituted indole 27b as a result from decreasing the electron density thru the substitutions.

The alkyl side chains have little effect on the absorption and fluorescence maxima's of the dyes as showed by dye 27a-g in Table 2.

<table>
<thead>
<tr>
<th>Dye</th>
<th>charge of methine carbon</th>
<th>Methine carbon shift (ppm)</th>
<th>Methine proton shift (ppm)</th>
<th>$\lambda_{abs}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27a</td>
<td>-0.626</td>
<td>82.78</td>
<td>6.31</td>
<td>537</td>
</tr>
<tr>
<td>27b</td>
<td>-0.584</td>
<td>83.57</td>
<td>6.31</td>
<td>556</td>
</tr>
<tr>
<td>27c</td>
<td>-0.635</td>
<td>84.00</td>
<td>6.31</td>
<td>564</td>
</tr>
<tr>
<td>27d</td>
<td>-0.632</td>
<td>83.93</td>
<td>6.25</td>
<td>562</td>
</tr>
<tr>
<td>27e</td>
<td>-0.668</td>
<td>83.69</td>
<td>6.29</td>
<td>564</td>
</tr>
<tr>
<td>27f</td>
<td>-0.632</td>
<td>83.69</td>
<td>6.34</td>
<td>565</td>
</tr>
</tbody>
</table>
An common characteristic observed with this set of dyes is their broad absorbance spectra that can be attributed to a high level of aggregation, as shown using compound 27f in Figure 11.²

Brooker et al. showed that if the basicity of the two nuclei is not equal the absorption would not be at the midpoint. The substitution at Y as shown in both Table 3 and Figure 12 position of the indole resulted in either a hypsochromic or bathochromic shift of absorbance maxima when comparing the absorption spectra of the dyes 27a which are reflected the change basicity, of the heterocycles in dyes 27a, 30a-c.
Table 3 Meso-proton shifts in relation to absorbance maxima in MeOH

<table>
<thead>
<tr>
<th>Y</th>
<th>$\lambda_{ab}$ (nm)</th>
<th>$\lambda_{em}$ (nm)</th>
<th>Stoke's shift</th>
<th>Charge of methine carbon</th>
<th>Methine carbon shift (ppm)</th>
<th>Methine proton shift (ppm)</th>
<th>N-CH$_3$ (ppm)</th>
<th>N-CH$_2$ (ppm)</th>
<th>Phase angle 1 (°)</th>
<th>Phase angle 2 (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30a</td>
<td>O</td>
<td>555</td>
<td>577</td>
<td>22</td>
<td>-0.531</td>
<td>87.40</td>
<td>6.48</td>
<td>4.37</td>
<td>4.16</td>
<td>175.52</td>
</tr>
<tr>
<td>30b</td>
<td>S</td>
<td>533</td>
<td>589</td>
<td>56</td>
<td>-0.248</td>
<td>74.91</td>
<td>6.15</td>
<td>4.48</td>
<td>4.05</td>
<td>-30.25</td>
</tr>
<tr>
<td>27a</td>
<td>C(CH$_3$)$_2$</td>
<td>537</td>
<td>592</td>
<td>55</td>
<td>-0.368</td>
<td>82.78</td>
<td>6.31</td>
<td>4.46</td>
<td>3.60</td>
<td>28.83</td>
</tr>
<tr>
<td>30c</td>
<td>C(CH$_3$)$_2$</td>
<td>553</td>
<td>591</td>
<td>38</td>
<td>-0.368</td>
<td>82.92</td>
<td>6.43</td>
<td>4.45</td>
<td>3.47</td>
<td>29.65</td>
</tr>
</tbody>
</table>

Figure 12 Absorbance spectra of select dyes in MeOH at 20 uM.

The benzoxazole heterocycle influenced the conjugated system shown by shifting in both $^1$H peak of the methine-proton, as shown in Table 3 and absorbance spectra as shown in Figure
The observed chemical shift of the methine-proton is most likely due to altering the electron density from the surrounding atoms. Oxygen is more electronegative than carbon, and therefore attracts more electronegativity from the methine chain corresponding to a $\lambda_{\text{max}}$ shift from 533 nm (30b) to 555 nm (30a) and a shift of methine-proton shift of 6.15 (30b) to 6.48 (30a) ppm as show in Figure 12 and Figure 13 respectively. Sulfur is only slightly more electronegative than carbon causing a smaller methine-proton shift from 6.15 (30b) to 6.30 (27a) ppm and corresponding a similar shift in $\lambda_{\text{max}}$ 533 nm 30b to 537 27a. The broad shape of the UV-Vis spectra are relative similar when compared to each other for the all 27a-g, 30a, and 30c; expect for 30b which shows a spectra with two local maxima’s, as shown in Figure 12. An increase in conjugation leads to bathochromic shift of the absorbance maxima and corresponding to a $\lambda_{\text{max}}$ to 542 as shown by 30c. Furthermore, the benz[e]indole heterocycle also shows the largest shift in both the meso-proton at 6.43 ppm and $\lambda_{\text{max}}$ both due to the lack of an electronegative element around the methine chain and increased conjugation in the heterocycle.

In addition, an increase in absorbance maxima lead to a decrease in $\varepsilon$. The energy transitions in cyanine dyes have been shown to be a dominant $\pi-\pi^*$ transition.\textsuperscript{44,45} The HUMO and LUMO orbitals for the dyes 27a, 30a-c are shown in Figure 13. The energy gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of compound 30c is the lowest among all of the synthesized monomethine, 6.60 eV. This is likely due to the increased conjugation around the benz[e]indole heterocycle which led to further delocalizing of the electrons making the compound more stable. Compound 30a with the benzoxazole heterocycle shows the highest energy gap likely due to both the lone pair of electrons and electronegativity of the oxygen atom. The second highest energy gaps arise from 30b, the benzothiazole heterocycle derivative due to the lone pairs of the sulfur atom followed by 27a. In the set of compounds, the HOMO orbitals is spread evenly throughout the compound, while the LUMO orbitals is mostly in benz[c,d]indole heterocycle, which suggests that there is a high electron density is on the benz[c,d]indole; This is also observed in the EMP of the dyes.
2.5 Conclusion

Monomethine cyanines were prepared with red shifted absorbance and fluorescence properties in comparison to previously synthesized asymmetric monomethines. The 10 derivatives that were synthesized in good yield have large Stokes shifts. Additionally, when these dyes are within a viscous environment, their fluorescence becomes observable which would allow for developing viscosity-dependent fluorescence measurements and direct analyses of various environments in biological systems\textsuperscript{46}. Future work would include observing the dyes’ interaction with nucleic acids.

2.6 Experimental

Synthesis of 1-butylbenzo[c,d]indol-2(1H)-one 21

Synthesis of 1-butylbenzo[c,d]indol-2(1H)-one (21) was performed by adding 40% sodium hydroxide (22.3 mmol, 0.7 mL) to a solution of the amide, benzo[c,d]indole-2(1H)-one (1.5 g, 8.9 mmol) in 15 mL of N-methyl-2-pyrrolidinone. Iodobutane (1.2 equiv) was then added dropwise to the reaction mixture, which was heated to 50°C for 1 h, after which no starting material was
visible on TLC. After cooling to room temperature, water (10 mL) was added, and the crude product was extracted with DCM and washed with brine. The organic layer was dried over anhydrous magnesium sulfate, gravity filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel using ethyl acetate/hexanes (1:10) as the eluting solvent.

Synthesis of 1-butylbenzo[cd]indole-2(1H)-thione 22

A mixture of amide 21 (200 mg) and diphosphorus pentasulfide (365 mg) in pyridine (8 mL) was refluxed for 2 h. The cooled reaction mixture was acidified with concentrated HCl and a red precipitate formed upon cooling. The crude product was filtered and dried. 1H NMR (300 MHz, DMSO-d6): d ppm 0.93 (t, J = 9.0 Hz, 3H), 1.39 (m, J = 9.0 Hz, 2H), 1.77 (m, J = 9.0 Hz, 2H), 4.35 (t, J = 9.0 Hz, 2H), 7.46 (d, J = 6.0 Hz, 1H), 7.61 (t, J = 6.0 Hz, 1H), 7.77–7.84 (m, 2H), 8.15 (d, J = 6.0 Hz, 1H), 8.24 (d, J = 9.0 Hz, 1H).

Synthesis of 1-butyl-2-(methylthio)benzo[cd]indol-1-ium iodide 23

A mixture of compound 22 and iodomethane (14 equiv) in the absence of solvent was heated to reflux for 8 h. The iodomethane was removed in vacuo. The resulting residue was treated with diethyl ether, and the mixture was sonicated for 30 min and was suction filtered, washed with diethyl ether, and dried leaving an 82% yield. 1H NMR (400 MHz, CDCl3): d ppm 0.82 (t, J = 7.6 Hz, 3H), 1.39 (q, J = 7.6 Hz, 2H), 1.82 (m, J = 7.2 Hz, 2H), 4.51 (t, J = 7.2 Hz, 2H), 7.72 (t, J = 8.0 Hz, 1H), 7.99 (t, J = 7.6 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 7.6 Hz, 1H), 8.50 (d, J = 7.6 Hz, 1H), 8.99 (d, J = 8.0 Hz, 1H). 13C NMR (100 MHz, CDCl3): d ppm 12.73, 17.73, 19.16, 29.47, 47.41, 117.02, 121.76, 127.29, 127.97, 128.12, 128.83, 130.47, 135.05, 136.93, 137.32, 171.74

General Synthetic Procedure for the indolium salts

Compounds 26a-g were synthesized as previously reported. Each compound was then dissolved in acetonitrile (25 mL) and 4 molar equivalences (eq.) of the primary alkyl halide were added to the solution. The reaction mixture was then refluxed at 90° C for 72 h. Thin layer
chromatography (TLC) was used to monitor the reaction in a solution of 4:1, DCM:hexanes. Upon cooling the reaction mixture to room temperature acetone was added followed by diethyl ether to precipitate the salt. The solid was filtered and washed with diethyl ether. The salts were used without further purification in subsequent reactions.

Synthesis of 5-Bromo-2,3,3-trimethyl-1-(3-phenylpropyl)-3H-indol-1-ium bromide (26g)

Compound 26 (0.02 mmol) was dissolved acetonitrile (25 mL) and to the solution 1-bromo-3-phenylpropane (0.04 mmol, 20 mL) was added. The reaction mixture was then refluxed at 90° C for 72 h. Thin layer chromatography (TLC) was used to monitor the reaction using DCM:hexane (4:1) as eluting solvent. The reaction mixture was allowed to cool to room temperature; acetone was added to the mixture, followed by diethyl ether to precipitate the salt. The solid was collected via vacuum filtration and washed with diethyl ether to afford 26g as a dark brown solid. Yield 43%, 3.5 g, mp 160-161° C; 1H NMR (400 MHz, CDCl3): 1.60 (s, 6H), 2.29 (m, J = 8.0 Hz, 2H), 2.88 (t, J = 8.0 Hz, 2H), 3.03 (s, 3H), 4.79 (t, J = 8.0 Hz, 2H), 7.20 (d, J = 4.0 Hz, 3H), 7.29 (d, J = 4.0 Hz, 2H) 7.50 (d, J = 8.0 Hz, 1H), 7.64 (d, J = 8.0 Hz, 2H) δ ppm 13C NMR (100 MHz, CDCl3): δ ppm 16.65, 23.17, 29.40, 32.57, 49.06, 54.83, 117.30, 124.47, 126.74, 126.90, 128.59, 128.96, 132.80, 139.57, 140.17, 143.62, 196.46.

Synthesis of 1-butylbenzo[c,d]indol-2(1H)-one (21) was performed by adding 40% sodium hydroxide (22.3 mmol, 0.7 mL) to a solution of benzo[c,d]indole-2(1H)-thione (1.5 g, 8.9 mmol) in 15 mL of N-methyl-2-pyrrolidinone. Iodobutane (1.2 mol eq.) was then added dropwise to the reaction mixture which was heated to 50 °C for 1 h, after which no starting material was present on TLC. After cooling to room temperature, water (10 mL) was added and the crude product was extracted with dichloromethane and washed with brine. The organic layer was dried over anhydrous magnesium sulfate, gravity filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel using ethyl acetate:hexanes (1:10) as the eluting solvent.

Synthesis of 1-butylbenzo[c,d]indole-2(1H)-thione (22). A mixture of amide (200 mg) and
diphosphorous pentasulphide (365 mg) in pyridine (8 mL) was refluxed for 2 h. The cooled reaction mixture was acidified with concentrated HCl and a red precipitate formed upon cooling. The crude product was filtered and dried. $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ ppm 0.93 (t, $J = 9.0$ Hz, 3H), 1.39 (m, $J = 9.0$ Hz, 2H), 1.77 (m, $J = 9.0$ Hz, 2H), 4.35 (t, $J = 9.0$ Hz, 2H), 7.46 (d, $J = 6.0$ Hz, 1H), 7.61 (t, $J = 6.0$ Hz, 1H), 7.77-7.84 (m, 2H), 8.15 (d, $J = 6.0$ Hz, 1H), 8.24 (d, $J = 9.0$ Hz, 1H).

Synthesis of 1-butyl-2-(methylthio)benzo[c,d]indol-1-ium iodide (23). A mixture of compound 3 and iodomethane (14 mol. eq.) in the absence of solvent was heated to reflux for 8 h. The iodomethane was removed in vacuo. The resulting residue was treated with diethyl ether and the mixture was sonicated for 30 minutes and was suction filtered, washed with diethyl ether, and dried leaving an 82% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 0.82 (t, $J = 8.0$ Hz, 3H), 1.39 (m, $J = 8.0$ Hz, 2H), 1.82 (m, $J = 8.0$ Hz, 2H), 4.51 (t, $J = 8.0$ Hz, 2H), 7.72 (t, $J = 8.0$ Hz, 1H), 7.99 (t, $J = 8.0$ Hz, 1H), 8.10 (d, $J = 8.0$ Hz, 1H), 8.21 (d, $J = 8.0$ Hz, 1H), 8.50 (d, $J = 8.0$ Hz, 1H), 8.99 (d, $J = 8.0$ Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 12.73, 17.73, 19.16, 29.47, 47.41, 117.02, 121.76, 127.29, 127.97, 128.12, 128.83, 130.47, 135.05, 136.93, 137.32, 171.74

General Synthesis of the monomethine dyes

Compound 23 was dissolved in 10 mL of CH$_3$CN and 1.5 mol. eq. each, of the appropriate indolium salt and triethlyamine was added to the solution. The reaction mixture was refluxed at 60° C for 1 hr. UV-Vis was used to monitor the reaction. Upon cooling to room temperature, diethyl ether was added to the reaction mixture to precipitate the dye. The solid was collected and washed with deionized water and diethyl ether. The dyes were purified via recrystallization by solvation in a minimal amount of methanol and dilution with diethyl ether causing precipitation.

(E)-1,3,3-trimethyl-2-((1-methylbenzo[cd]indol-2(1H)-ylidene)methyl)-3H-indol-1-ium iodide 27a (lo 5)$^1$H NMR (400 MHz, DMSO-d$_6$) d ppm 0.95 (t, $J=7.08$ Hz, 3 H) 1.45 (sxt, $J =$
7.30 Hz, 2 H) 1.65 (s, 6 H) 1.86 (quin, J = 7.30 Hz, 2 H) 3.47 (s, 3 H) 4.45 (t, J = 7.50 Hz, 2 H)
6.31 (s, 1 H) 7.44 (t, J = 7.34 Hz, 1 H) 7.51 - 7.63 (m, 2 H) 7.69 - 7.85 (m, 4 H) 7.88 - 7.96 (m, 2 H)
8.37 (d, J = 8.02 Hz, 1 H): \(^{13}\text{C NMR (100 MHz, DMSO-}\text{d}_6\}): XX

(E)-1-Butyl-2-((1-butyl-3,3-dimethylindolin-2-ylidene)methyl)benzo[c,d]indol-1-ium iodide (27b); Yield 66 %, 0.48 g; mp 220 - 221 °C; \(^1\text{H NMR (400 MHz, DMSO-}\text{d}_6\): \(\delta\) ppm 0.62 (t, J = 8.0 Hz, 3H), 0.91 (t, J = 8 Hz, 3H), 0.99 (m, 2H), 1.40 (m, 2H), 1.64 (s, 8H), 1.80 (m, J = 8.0 Hz, 2H), 4.18 (s, 2H), 4.49 (s, 2H), 6.31 (s, 1H), 7.41 (t, J = 4.0 Hz, 1H), 7.73 (m, 4H), 7.91 (m, 2H), 8.00 (d, J = 4.0 Hz, 1H), 8.35 (d, J = 8.0 Hz, 1H); \(^{13}\text{C NMR (100 MHz, DMSO-}\text{d}_6\): \(\delta\) ppm 12.94, 13.35, 18.34, 19.15, 25.37, 28.89, 29.53, 43.27, 49.78, 51.32, 83.57, 110.71, 113.99, 122.82, 122.95, 123.46, 126.05, 128.14, 129.22, 129.67, 132.41, 140.24, 140.35, 141.44, 155.54, 180.08; \(\lambda_{\text{abs}}\) = 556 nm in MeOH.

HRMS (ESI) calculated for [C\(_{30}\)H\(_{35}\)N\(_2\)]\(^{1+}\) m/z 423.2795, found m/z 423.1447

(E)-1-Butyl-2-((1-butyl-5-chloro-3,3-dimethylindolin-2-ylidene)methyl)benzo[c,d]indol-1-ium iodide (27c); Yield 34%, 0.40 g; mp 197 - 198 °C; \(^1\text{H NMR (400 MHz, DMSO-}\text{d}_6\): \(\delta\) ppm 0.62 (t, J = 8.0 Hz, 3H), 0.91 (t, J = 8 Hz, 3H), 0.99 (m, 2H), 1.40 (m, 2H), 1.59 (m, 2H), 1.64 (s, 8H), 1.81 (m, 2H), 4.14 (m, 2H), 4.52 (m, 2H), 6.30 (s, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.73 (m, 2H), 7.90 (d, J = 8.0 Hz, 1H); \(^{13}\text{C NMR (100 MHz, DMSO-}\text{d}_6\): \(\delta\) ppm 13.26, 13.66, 18.64, 19.46, 25.65, 29.03, 29.93, 43.75, 50.28, 51.58, 84.00, 111.73, 115.66, 123.55, 123.66, 123.81, 128.84, 129.00, 129.13, 129.27, 129.56, 129.98, 130.09, 130.62, 133.14, 140.52, 140.83, 142.59, 156.40, 179.80; m.p. 197-198 °C; \(\lambda_{\text{abs}}\) = 562 nm in MeOH. HRMS (ESI) calculated for [C\(_{30}\)H\(_{34}\)ClN\(_2\)]\(^{1+}\) m/z 458.0568, found m/z 458.7525

(E)-1-butyl-2-((1-butyl-5-methoxy-3,3-dimethylindolin-2-ylidene)methyl)benzo[c,d]indol-1-ium iodide (27d); Yield 50%, 0.24 g, mp 174-175 °C; \(^1\text{H NMR (400 MHz, DMSO-}\text{d}_6\): \(\delta\) ppm 0.62 (t, J = 8.0 Hz, 3H), 0.90 (t, J = 8.0 Hz, 3H), 1.05 (m, J = 8.0 Hz, 2H), 1.39 (m, J = 8.0 Hz, 2H), 1.63 (s, 8H), 1.85 (t, J = 8.0 Hz, 2H), 3.86 (s, 3H), 4.18 (t, 2H), 4.43 (t, J = 8.0 Hz, 2H), 6.25
(s, 1H), 7.10 (d, J = 8.0 Hz, 1H), 7.41 (s, 1H), 7.62-7.71 (m, 3H), 7.81-7.85 (m, 3H), 8.30 (d, J = 8.0 Hz, 1H); $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ ppm 13.49, 13.92, 18.84, 19.72, 25.63, 29.54, 29.96, 43.54, 50.38, 52.18, 56.16, 83.93, 109.65, 110.11, 114.05, 115.69, 122.63, 124.24, 127.56, 129.55, 129.76, 130.15, 130.20, 132.29, 135.10, 141.17, 142.92, 154.81, 159.08, 180.29; $\lambda_{abs}$ = 564 nm in MeOH. HRMS (ESI) calculated for [C$_{31}$H$_{37}$ClN$_2$O]$^{1+}$ m/z 453.2900, found m/z 453.2441.

(E)-2-((5-Bromo-1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)-1-butylbenzo[c,d]indol-1-ium iodide

(27e); Yield 25%, 0.12g, mp 195-196°C; $^1$H NMR (400 MHz, DMSO-d$_6$): δ ppm 0.93 (t, J = 8.0 Hz, 3H), 1.10 (t, J = 8.0 Hz, 3H), 1.41 (m, 2H), 1.64 (s, 6H), 1.84 (t, J = 8.0 Hz, 2H), 4.17 (m, 2H), 4.49 (t, J = 6.0 Hz, 2H), 6.29 (s, 1H), 7.62 (d, J = 12.0 Hz, 1H) 7.73-7.81 (m, 3H), 7.88-7.95 (m, 2H), 8.02 (s, 1H), 8.13 (d, J = 4.0 Hz, 1H), 8.40 (d, J = 4.0 Hz, 1H); $^{13}$C NMR (100 MHz, MeOD-d$_4$): δ ppm 12.66, 13.49, 13.65, 20.44, 25.49, 30.91, 72.81, 83.69, 84.64, 112.53, 115.69, 120.11, 124.92, 124.98, 127.00, 129.13, 130.07, 130.53, 131.02, 132.22, 134.07, 141.26, 141.26, 141.78, 143.44, 158.41, 179.99; $\lambda_{abs}$ = 564 nm in MeOH; HRMS (ESI) calculated for [C$_{28}$H$_{30}$BrN$_2$]$^{1+}$ m/z 473.1567, found m/z 473.0529.

(E)-2-((5-Bromo-1-butyl-3,3-dimethylindolin-2-ylidene)methyl)-1-butylbenzo[c,d]indol-1-ium iodide (27f); Yield 54%; 0.46g, mp 187-186°C; $^1$H NMR (400 MHz, DMSO-d$_6$): δ ppm 0.59 (s, 3H), 0.90 (s, 3H), 0.97 (m, 2H), 1.38 (m, 2H), 1.57 (s, 6H), 1.67 (s, 6H), 1.82 (m, 2H), 4.13 (s, 2H), 4.54 (s, 2H), 6.34 (s, 1H), 7.67-7.77 (m, 4H), 7.90 (m, 2H), 8.02 (s, 1H), 8.06 (d, J = 4.0 Hz, 1H), 8.37 (d, J = 8.0 Hz, 1H); $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ ppm 12.78, 13.19, 18.19, 18.98, 25.27, 28.55, 29.47, 43.44, 48.09, 49.84, 51.14, 83.69, 111.33, 115.64, 118.25, 123.09, 123.34, 125.84, 128.58, 129.05, 129.43, 129.66, 130.69, 132.64, 139.95, 140.78, 142.41, 155.95, 179.08; $\lambda_{abs}$ = 565 nm in MeOH; HRMS (ESI) calculated for [C$_{30}$H$_{34}$BrN$_2$]$^{1+}$ m/z 502.5078, found m/z 502.7264.

(E)-2-((5-Bromo-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)methyl)-1-
butylbenzo[c,d]indol-1-i um iodide (27g); Yield 32 %, 0.29 g, m.p. 201-202°C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) ppm 0.92 (t, \(J = 8\) Hz, 3H), 1.40 (m, 2H), 1.65 (s, 6H), 1.74 (m, 2H), 1.84 (m, 2H), 2.40 (m, 2H), 4.10 (m, 2H), 4.41 (m, 2H), 6.19 (s, 1H), 6.76 (m, 5H), 7.66 (d, \(J = 4\) Hz, 1H) 7.68 (q, \(J = 8\) Hz, 2H), 7.78 (s, 2H), 7.88 (t, \(J = 6\) Hz, 1H), 7.98 (m, 1H), 8.02 (s, 1H), 8.08 (d, \(J = 8\) Hz), 8.40 (d, \(J = 4\)Hz, 1H) \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) ppm 13.21, 19.02, 25.29, 28.31, 29.54, 30.99, 49.11, 51.02, 83.12, 111.50, 115.30, 118.11, 123.11, 123.55, 124.96, 125.83, 126.4, 127.13, 127.22, 127.92, 128.80, 129.00, 129.38, 130.69, 132.74, 139.37, 139.81, 141.02, 142.32, 155.95, 178.11; \(\lambda_{ab s}\) = 567 nm in MeOH; HRMS (ESI) calculated for [C\(_{35}\)H\(_{36}\)BrN\(_2\)]\(^{1+}\) m/z 563.2056, found m/z 563.0953.

1-butyl-2-((3-methylbenzo[d]thiazol-2(3H)-ylidene)methyl)benzo[cd]indol-1-ium iodide (30a); Yield 57 %; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) ppm 0.96 (t, \(J = 7.07\) Hz, 3H) 1.37 - 1.57 (m, 2H) 1.74 - 1.94 (m, 2H) 4.05 (s, 3H) 4.48 (t, \(J = 6.57\) Hz, 2H) 6.15 (br. s., 1H) 7.56 - 7.68 (m, 3H) 7.73 (t, \(J = 7.58\) Hz, 1H) 7.80 - 7.92 (m, 2H) 8.04 (t, \(J = 7.71\) Hz, 1H) 8.15 (d, \(J = 7.58\) Hz, 1H) 8.40 (d, \(J = 8.08\) Hz, 1H) 9.17 (d, \(J=7.07\) Hz, 1H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) ppm 13.96, 19.49, 29.66, 31.41, 43.09, 74.91, 109.49, 111.74, 112.01, 122.36, 125.99, 126.65, 127.91, 129.09, 129.17, 129.67, 129.84, 131.26, 132.33, 140.51, 146.24, 154.99, 161.40

1-butyl-2-((3-methylbenzo[d]oxazol-2(3H)-ylidene)methyl)benzo[cd]indol-1-ium iodide (30b); Yield 69 %\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) ppm 0.80 - 1.11 (m, 3H) 1.46 (d, \(J = 6.57\) Hz, 2H) 1.82 (br. s., 2H) 4.16 (br. s., 3H) 4.37 (br. s., 2H) 6.48 (br. s., 1H) 7.55 (d, \(J = 6.82\) Hz, 1H) 7.67 (dd, \(J = 19.96, 7.07\) Hz, 2H) 7.72 - 7.83 (m, 2H) 7.88 (t, \(J = 7.58\) Hz, 1H) 8.04 (d, \(J=8.08\) Hz, 1H) 8.21 (d, \(J = 7.33\) Hz, 1H) 8.32 (d, \(J=8.08\) Hz, 1H) 9.24 (d, \(J = 7.07\) Hz, 1H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) ppm 14.23, 20.13, 30.07, 35.81, 43.79, 87.40, 109.46, 115.35, 122.44, 124.02, 125.07, 125.73, 127.17, 127.33, 129.23, 128.65, 130.06, 130.12, 132.73, 141.43, 141.63, 154.71, 166.28

(E)-1-butyl-2-((1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)methyl)benzo[cd]indol-1-ium iodide (30c); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) d ppm 0.95 (t, \(J=7.34\) Hz, 3H) 1.45 (sxt, \(J =
3.1 Photodynamic, an Introduction

Methods for heliotherapy, (the therapeutic use of light) have been dated as far back as 1400 B.C and in 1903, the Nobel Prize in Physiology or Medicine was awarded to Niels Ryberg Finsen, for his methods utilizing light for the treatments lupus vulgaris (a skin disease). The light used in the treatments can originate either from an artificial source, natural source, or as a combination of both artificial and natural sources. The combination of light source and an external agent, usually photosensitizer, constitutes photochemotherapy. Photodynamic therapy (PDT) is a type of photochemotherapy which using light, a photosensitizer, and molecular oxygen for treatments. The selective accumulation of a photosensitizer at the target, such as cancer cells followed by excitation via an appropriate light source, is the mode in which PDT obtains its therapeutic properties. Photothermal therapy (PTT), in another form of phototherapy which causes cellular damage (hyperthermia) through the conversion of energy to heat by the photosensitizer from the absorbed photons. In general a photosensitizer is a molecule that is capable of absorbing light energy and transferring that energy to an adjacent molecules/system. A photosensitizer as a theranostic agent would have specific physical and photo-physical properties. The
Idea physical properties of the photosensitizer would include qualities that allow selectivity, low toxicity for both the photosensitizer and its metabolites and excretion from the body upon completion of treatment. The photo-physical properties of the model photosensitizer include absorption in the NIR therapeutic window (650–800 nm), high extinction coefficient ($\varepsilon >50,000–100,000\ M^{-1}\ cm^{-1}$) and treatment dependent properties, such as a high singlet oxygen quantum yield ($\Phi_D$) for PDT. FDA approved sensitizers for PDT are shown in Figure 14.

Figure 14 FDA approved sensitizer for PDT.

3.2 Photochemothreapy mechanism

Figure 15 Motifs for photosensitizers for PDT agents classes; absorbing unit in red
Although photosensitizer can range from very simple organic molecules such as ALA, to extended organometallic or supramolecular aggregates, they are described as a molecule that is efficient at absorbing photons and transferring that energy to other molecules with common organic motifs for photosensitizers includes furanocoumarin, porphyrinoid, anthraquinone, phthalocyanine, phenothiazine, bodipy and squaraine bases, as shown in Figure 15\textsuperscript{47,50,52}. The conjugation of in the photosensitizer agents allow for the compound to absorb light, where the energy transfer is called the photosensitized reaction. Both PTT and PDT utilizes the sensitizer in an excited state for the energy transfer to induce damage to cause damage to targets. A requisite for the energy transfer, is that the lifetime of the molecule in the excited state must be long enough for the reaction\textsuperscript{53,54}. When an organic molecule absorbs light, the absorbed photon promotes the molecule from a singlet ground state ($^1$PS) to a singlet excited state ($^1$PS*) as shown in Figure 16. This $^1$PS*) state can stabilize either via a radiative process such as the singlet–singlet emission called fluorescence, or via non-radiative intersystem crossing from the singlet to triplet state ($^3$PS*). The energy of the $^3$PS* species can also dissipated via the radiative triplet–singlet emission process, called phosphorescence. Another pathway for the excitation energy is for it to thermally dissipate via internal conversion.

![Adapted Jablonski Diagram to show PDT mechanism](image)

**Figure 16** Adapted Jablonski Diagram to show PDT mechanism; isc (intersystem crossing) ic (internal conversion) fl (fluorescence) abs (absorbance) ph (phosphorescence)

In PDT the photosensitizer is excited at specific wavelength, which then produces reactive oxygen species (ROS), such as singlet oxygen ($^1$O$_2$) or free radicals (·OH) by transferring the ab-
sorbed energy to surrounding oxygen molecules. The energy transfer can occur via two different pathways, Type I and Type II, as shown in Figure 16. Type I mechanisms are classified when ROS are generated thru secondary reactions (energy transfer thru an intermediate and then to O$_2$ to generate ROS). Type II mechanism are classified by the direct energy transfer from the photosensitizer to molecular oxygen (O$_2$). Nonetheless, both phototherapies utilize a photosensitizer that absorbs light to achieve selective cell death and tissue devastation either via an energy transfer (PDT) or energy conversion (PTT) for efficacy.$^{50}$

### 3.3 Logic of photosensitizing Agent

A large percentage of chemotherapeutic anticancer drugs are compounds that interact with DNA.$^{55}$ DNA fundamental role in biology is to store genetic information, thus its stability against external perturbations is essential yet selective DNA oxidation is the mode in which the photosensitizers in both PDT and PTT obtain theranostic properties.$^{50-54,56}$ The cellular damage is dependent on the properties of the photosensitizer in both excited states and location within the cell with the direct triplet energy transfer to the DNA nucleobases leading to irreversible DNA lesions. The anatomy of a photosensitizer may be understood summarized into two parts: the chromophore and its peripheral modifications. The first grants both light absorption/emission and photosensitizing properties, while the later are important to their physical properties that affect both drug distribution and uptake such as lipophilicity. Among the many conjugated systems, cyanine dyes have been adapted to assume properties that would enable it to selectively target and assume absorbance/fluorescence bands in the desired therapeutic NIR window 600–800 nm range.

Example of photosensitized DNA cleavage have been reported for both monomethine dyes based on derivatives of YO and TO for both ss-DNA and ds-DNA but their short wavelength bands limit their therapeutic development.$^{57,58}$ Furthermore, the Henary lab has synthesized a series Meso-substituted pentamethine with benz[e]indolium terminal heterocycles were shown
that oxidatively cleave U19 plasmid DNA\textsuperscript{21}. Using this finding, the photosensitizing dyes would incorporate the pentamethine, to provide model photo physical properties such as absorbance in the near-infrared (>650 nm) region of the electromagnetic spectrum\textsuperscript{13}. This in return would allow for lower energy excitation sources to be during treatments. With the collaboration with Dr. Grants Lab, the synthesized dyes were evaluated for the photo-induced cleaving activity against pU19 plasmid DNA. The prospective photosensitizing dyes would incorporate phenanthridine and quinoline, as the terminal heterocycles in the cyanine system. Also, to study the effect of the polymethine chain, the selected heterocycles would also be synthesized in the trimethine fashion. The phenanthridine and quinoline core were outlined from Ethidium bromide, a well-documented nucleic stain, as shown in Figure 17. The assumption is made that the flat cationic heterocycles would promote stronger interaction with DNA.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Structure of Ethidium bromide with the phenanthridine core outlined in red and quinoline in blue.}
\end{figure}

\section*{3.4 Synthesis of Photosensitizing Polymethine Dyes}

Two sets of dyes were synthesized, with the first set consisting of dyes with phenanthridine, as shown in Scheme 7. The synthetic preparation of the symmetric polymethine cyanine dye consists of the condensation of two salts, quaternary salt \textbf{34} and a polymethine agent (\textbf{37} or \textbf{38}). Synthesis of the first part \textbf{34}, began with formation of the 6-methylphenanthridine \textbf{33} which was isolated in two steps. The amide \textbf{32}, afforded by the acetylation of the 2-aminobiphenyl \textbf{3} with
acetic anhydride (Ac₂O) and was proceeded by the dehydrative ring closure via the Morgan-Walls reaction with phosphoryl chloride (POCl₃) under nitrogen. A saturated solution of sodium carbonate (Na₂CO₃) was used to neutralize the reaction mixture due to the increase of acidic byproduct from the cyclization and to ensure the of the phenanthridine nitrogen for subsequent reactions.

Scheme 7 Synthesis of novel Phenatridine containing dyes; 39 Commercial reagent
After isolation of the phenanthridine ring 33 thru extraction, various alkyl halides were used for quaternizing the N-phenanthridinyl to afford the salts, 34a-c. The N-methyl salt 34a was used to prepared trimethine dye 36 via the base catalyzed condensation with triethyl ortho formate (CH(OEt)₃) in Ac₂O. The pentinthein dyes 35a-f where synthesized with altering substitution on the pentamethine chain. The halogenated polymethine precursors were generated by reacting aniline with either mucoclorhlc 38a or mucobromic 38b acid in ethanol with light heating. The base catalyzed condensation was then proceed between the prepared phenantrhindinium salts with either the commercially available polymethine reagent, 39 or synthesized the halogenated
polymethine reagents $38a$ or $38b$. Trimethylamine (TEA) was used as the base in the condensation reaction to afford the polymethine dyes. The base in reaction caused the deprotonation of the methyl at the 6 position and acetic anhydride catalyzed the reaction.

Scheme 8 Synthesis of novel 4-quinyl containing dyes

The second set of series of dyes, shown in Scheme 8 were prepared from the commercially available 4-methyl quinoline. The quinoline was alkylated in acetonitrile with the appropriate halide to afford the salts $40a$-$c$. The second step was proceeded by base-catalyzed condensation of salts with either polymethine linker $38$ or $39$ in acetic anhydride with TEA, to afford the pentamethine dyes, $42a$-$g$. The trimethine dye $41$ was synthesized by the condensation of quaternary salt $40a$ with equal mols of TEA as base in triethylortho formate (CH(OEt)$_3$). Both sets of dyes were either purified via column chromatography using DCM:MeOH solvent mixture or selective precipitation from methanol in ether. Both $^1$HNMR and $^{13}$CNMR was used to structurally analyze the dyes synthesized.

3.4.1.1.1 Molar Absorptivity

The absorption spectra were recorded on a Varian Cary 3G UV-Visible Spectrophotometer interfaced with Cary WinUV Scan Application v3.00 at varying concentrations (µM) in cuvettes with a pathlength 1 cm. For each optical measurement, stock solutions were prepared by adding the appropriate mass of each individual compound on a five digit analytical balance to make a
1.0 mM solution. From these stock solutions, the dilutions seen in appendix were obtained to determine the molar absorptivity values in methanol.

3.4.1.2 Computational methods

The Calculated LUMO and HOMO orbitals where obtained using a restricted hybrid HF-DFT SCF calculation performed using Pulay DIIS + Geometric Direct Minimization and 6-31G* basis set, B3LYP Method via Spartan or Gassuioin(03).

3.5 Characterization

Compound 35c was chosen as a representative for characterization and analysis of set of pentamethine dyes 35. The 1H NMR spectra of compound 35f measured in DMSO-d6 can be seen in Figure 18. The structure of the dye can be classified into two parts, the phenanthridine terminal and the poly-methine bridge. The phenanthridine ring was colored based on the nomenclature of heterocyclic compounds, benzo[c]quinoline and the polymethine chainin in green in Figure 18. There are five protons on the polymethine chain and sixteen protons on the heterocyclic ring system, yet the symmetrical nature of the dye allows for two equivalent proton of all protons except the proton of the center carbon. Therefore, the dye would have 15 protons peaks that would appear in the spectra, eleven in the aromatic region and four in the aliphatic region of the spectra, respectively.
Figure 18. $^1$H NMR spectrum of 35f in DMSO-d6 at 25°C

The spectra in Figure 19 shows eight peak signals in the aromatic region and four in the aliphatic region of the spectra. The polymethine chain would produce two triplets and a doublet and would account for three peaks in the spectra. The phenanthridine (benzo[c]quinoline) ring would produce a splitting pattern of would result in both four doublets and triplets, for a total of eight signals but the $^1$H NMR only of 35f in either solvent, (DMSO-$d_6$ or CDCl3-$d$) produced less than eight signals that would correspond to the ring system due to the overlapping of peaks as shown in Figure 21.
An effort was made to correlate the protons signals to the structure of dye 35f as the proton assignment found in Figure 19, in which each colored part of structure would tier respective peak that are coupled based on integration, splitting pattern and coupling constant (J [Hz]). The three furthest peaks up-field in DMSO, have both similar and large coupling values (J >12 Hz) when compared to signals further downfield (J < 9 Hz). The integration, splitting patterns and similar coupling constant (J [Hz]) of the peaks at 6.75(d), 6.83 (t), 7.46(t) ppm would corresponds to five protons of the methane chain, labelled in green. The CDCl₃-d spectra also show three peaks with large and similar coupling constant, and are also labeled in green in Figure 19. The HOMO and LUMO orbitals as shown in Figure 20, reflect the alternating charges on the carbon atoms in the methine chain. the calculated charges of the carbon also show alternating net charges spread across the dye.
Further analyzing the $^1$HNMR spectrum in DMSO-$d_6$, in Figure 21, two are the peaks with coupling values larger than 8 Hz suggesting that they are part of the same environment and were labeled in blue. The triplet at 8.63 ($J = 8.11$ Hz) ppm integrates total of four protons, suggesting that there are two protons from the ring. The multiplet from 7.72-7.78 ppm appears be the result of two doublets due to the space between the peaks (8 Hz) being smaller than the coupling constant of the peaks, $J = 9.39$ Hz; the large coupling constant suggests that it belong to the ring in blue. The doublet at 8.23 ($J = 7.85$ Hz) and triplet 7.74 ($J = 7.68$ Hz), at would correspond to two protons from the ring labeled in red, due to having similar coupling constant. The remaining protons from the ring in red are account for in multiplet from 7.89-7.96 ppm, which
integrates to two protons; the peak shape appearing to be the result of two overlapping peaks and would account for the other two proton of the ring in red.

Analyzing the $^1$H NMR spectrum in CDCl$_3$-d in Figure 21, the peak furthest downfield integrates to four protons, indicating two protons. This doublet at 8.32 ($J = 7.83$ Hz) and the triplet at 7.81 ($J = 7.58$ Hz) have a similar coupling value, similar the ring in red, as established in the DMSO spectra. The integration of these two peak accounts for to two from the ring in red. The multiplet at 7.41 -7.47 integrates to four protons and appear to be a doublet and triplet. However, the different coupling values suggest that these are two protons from different rings, 6.32 vs 13.14 Hz; one from the methane chain and the other from the ring in red as the last peak from the ring.

When comparing the aromatic region of 36f CDCl$_3$-d spectra showed a doublet with a coupling constant of 8.1 Hz, indicating that it belongs to the ring colored in blue. The multiplet 7.64-7.70 ppm integrates to six proton which indicate other three proton of the ring in blue since the other protons have already be assigned.

The overall structure of the dyes with the phenanthridine is bent as shown in Figure 22 when compared to the quinoline dyes which are flat as shown in Figure 23a. The bent shape of the molecule could due to both the electrostatic repulsion and steric hinderence from the hydrogens of the polymethine chain and phenanthridine ring. Nonetheless, the HOMO and LUMO show similar electron density across the polymethine chain as shown in Figure 23b.
As such, dye 42d was chosen for complete characterization, as representative example for dyes 42. The structure of the dye 42d can be classified into two parts, the quinoline terminal and the poly-methine bridge, thus there are five protons on the polymethine chain and twelve protons on the heterocyclic ring system. The symmetrical nature of the dye allows for two equivalent proton of all protons except the proton of the center carbon. Thus the dye would have eleven protons peaks that would appear in the spectra, nine in the aromatic region and two in the aliphatic region of the spectra, respectively. The $^1$H NMR spectra of compound 42b measured in DMSO-$d_6$ can be seen in Figure 25. In Figure 26 an attempt was made to assign the protons signals in the aromatic region of the $^1$HNMR spectra of the dye 42d.
Figure 251HNMR spectra of the dye 42b.

The proton signal assignment were based on integration, splitting patterns and electron density; the colored labeling found in in Figure 26 was based on the peaks similar coupling constant, J [Hz]. There are five protons on the polymethine chain and twelve protons on the heterocyclic rings. The symmetrical nature of the dyes causes two equivalent of every proton except the proton of the center carbon and results nine distinct proton.

Figure 26 proposed 1H labeling of 42b in DMSO-d6

The quinoline is connected to the methine chain at 4 position, resulting in two doublets from one side of the ring (in blue) and a sets of both triplet and doublet, from the other side in red, as
shown in Figure 28. Following the molecular structure labeling in Figure 26, the two peak furthest down-field have different coupling values, suggesting that they belong to different ring in the heterocycle. The first doublet at 8.36 ppm ($J = 8.34$ Hz) has a different coupling value from second doublet at 8.09 ppm ($J = 7.07$ Hz) has a coupling constant of. Another doublet at 7.32 ppm ($J = 7.07$ Hz), also has a coupling constant and would belong to the heterocycle colored red A. The peaks at 7.95, 6.93, 6.63 have large coupling values ($J > 12$ Hz) the integration of these three peaks corresponds to five protons of the methane bridge colored in green. The multiplet ranging from 7.78-7.87 ppm appear to be an overlap of a doublet and triplet and with an integration that would correspond to two protons colored red. The triplet at 7.53 would account for the forth signal from the ring in red as it integrates to one proton on the red heterocyclic ring.

Figure 27 Time-dependent UV-Vis Spectra of 36f in DMSO

When you compare the two pentamethine dye systems, dyes 36f produces a very board absorbance spectra as shown in Figure 27. Strong self-aggregation tendency of polymethine cyanine dyes caused by Van der Waals forces and π-π conjugated system interaction. In addition, the conjugated system will be broken due to autoxidation, resulting in abs bleach. In addition to this the dyes observes solvatochromic shifts, as shown in Table 4. For example when any of the dyes is present in methanol, solution is blue but in dichloromethane, the solution is green. This effect was not seen in the other set of dyes. After the characterization of the
dyes sets, the compounds were sent to Dr. Grant’s lab to be tested for photo-cleavage (PC) properties with pUC19 DNA from *E.coli* carried at physiological PH (7.4) and temperature (37°C).

Table 4 Photo physical properties of dyes 35a-f.

<table>
<thead>
<tr>
<th>Dye</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; in DCM (nm)</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; in MeOH (nm)</th>
<th>ε (Lmol&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt; x 10&lt;sup&gt;3&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>35a</td>
<td>736</td>
<td>725</td>
<td>37.9</td>
</tr>
<tr>
<td>35b</td>
<td>NR</td>
<td>718</td>
<td>48.8</td>
</tr>
<tr>
<td>35c</td>
<td>NR</td>
<td>722</td>
<td>40.8</td>
</tr>
<tr>
<td>35d</td>
<td>724</td>
<td>716</td>
<td>36.2</td>
</tr>
<tr>
<td>35f</td>
<td>726</td>
<td>713</td>
<td>39.8</td>
</tr>
</tbody>
</table>

3.6 Results and Discussion

For DNA photosensitization, the absorption of light and the subsequent photochemical pathways are mediated by the interacting chromophore; with three main modes of complexation have been characterized for canonical B-DNA: (1) the groove binding (2) intercalation (3) insertion. For example, the groove binding interactions are stabilized by electrostatic interactions between the negatively charged backbone and cationic photosensitizers. In the case of ligand intercalation, the π-stacking interaction strongly compensates for the helical distortion in B-DNA helical structural. Aromatic ring system, such as that of cationic Ethidium bromide, can diffuse into the hydrophobic area between the two DNA base pairs. Lastly, if insertion is the mode interface with DNA energy is offset by the non-Watson-Crick pairing, in which one sensitizer ejects one of the nucleo bases from take its place. The dyes screened are shown in Figure 29 with their respective photo-cleavage (PC) activity. In the study, dye 36f showing the most damage to the DNA, following by 42a and 42d. The dyes have the potential of interacting through groove binding to DNA due to the bent shape and charge of the dye. Intercalating would also be possible if one on the end terminals of the dyes where to diffuse between the two DNA base pairs. In each described case, the mode of interaction with the DNA and action of each dye could be different as the interactions modes are competitive and several of them may coexist. Additional
experiment need to be conducted to probe the mode of DNA-complexation, in order to identify how the triplet energy is transferred, either directly to DNA or to molecular oxygen.

Figure 28 DNA Photo-oxidation Cleavage assay of select dyes, performed in triplicate; control = DNA and no dye
Reviewing the cleaving data in Figure 28, a general trend was observed for both sets pentamethine dyes in which the dyes with longer alkyl groups on the heteroatom of the ring, showed higher a cleaving activity when compared to same dye with a shorter chain. In addition, halogen substituent at the center of the pentamethine chain, decreased the PC activity, with the exception of 42g which has a longer chain (butyl) when compared to other dyes with halogen substitution. This suggests that strics play an part in the cleaving activity. Furthermore, shortening the polymethine chain decreased the PC activity, as in the case of the trimethine dyes 36 and 41.

The dye with the highest activity 35f, was then exposed to different reaction conditions further to investigate mode of induced cleavage. As shown in Figure 29 dye 35f showed that the induced cleavage was temperature dependent and is enhanced with the addition of biological metals such as, Fe^{3+} and Cu^{2+}.

![Image](51.png)

Figure 29 Both exposed to minimal light A) Agarose Gel Electrophoresis of Temperature dependent study of 35c at 10 μM with a reaction time of 24h; B) Agarose Gel Electrophoresis of 35f at 10 μM with addition of [Fe^{3+}] or [Cu^{2+}] at 10 μM with a reaction time of 24h at 37\(^{\circ}\)C.

However, during the experiments, dye 35f showed cleavage even when light exposure is minimized (in dark). Due to the finding, an additional set of experiments was conducted by the Grant Lab were The cleavage was also observed was time dependent as shown by Figure 30. Selected fragments of the terminal phenanthridine showed no cleavage induced in the presence with DNA suggesting that the polymethine system is needed for PC activity.
Figure 30 Agarose Gel Electrophoresis of 35f and pUC19 DNA. Conditions 1.5% agarose non-denaturing gel 10 mM sodium phosphate buffer (pH 7), minimal light exposure, 24 hr reaction time.

<table>
<thead>
<tr>
<th>ESS-16</th>
<th>Cont 1</th>
<th>Ess 1</th>
<th>Cont 2</th>
<th>Ess 2</th>
<th>Cont 3</th>
<th>Ess 3</th>
<th>Cont 4</th>
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</thead>
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<tr>
<td>Reaction time (Day)</td>
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<td>2</td>
<td>3</td>
<td>4</td>
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Figure 31 Selected fragments of the terminal DNA. Furthermore the dye still cleaves the plasmid DNA even with EDTA added to the reaction mixture as shown in Figure 31 suggesting that the dye alone is responsible for the PC. DNA binding can occur via three main ways, including intercalative, groove, and external binding modes. Stability of dye 35f in chelating agents improved the dyes stability in aqueous solutions as shown in Figure 33. Oxidation of the dye can occur if metal-complexation is formed in solution which would cause the dye to break down if oxidized by a metal.
Figure 32 Time dependent study of Dye 35f in various aqueous solution

The negatively charged DNA surface should attract these electron deficient system which could then diffuse into the hydrophobic area, such as between the DNA base pairs. Such a change in environment of the chromophore would be observed via its UV-Vis spectra, as in the case of DNA stain EtBr. Nonetheless when trying to observe the dye-DNA interaction, the spectra for 35f showed both absorption attenuation as a function of time in both sodium phosphate buffer and DNA solutions, shown in Figure 34. The bleaching in UV-vis absorption could attribute its' PC activity as photo-bleaching is dependent upon the molecular structure and the local environment

Figure 33 Time-Course UV-Vis Absorption of 35f in 10 mM Sodium Phosphate Buffer
In contrast, either triemthime dyes 36 or 41 containing the heterocycle as shown in Figure 35, which need a light source to induce cleavage.

Dye 36 in the presence of DNA has a red shift to $\lambda_{\text{max}}$ to about 600 nm and dye 41 exhibited hypochromic and bathochromic shifts in the presence of CT DNA with a $\lambda_{\text{max}}$ at around 710 nm. As shown in Figure 37.
3.7 Conclusion

The dyes were synthesized in good yield. The phenanthridine and quinoline core provide interaction with pDNA that induced cleavage when with light (35f). Utilizing 4-methylquinoline allowed the both tri- and penta-methine dyes to absorb at longer wavelength and still maintained the in PC activity for DNA in comparison to the other cyanine dyes synthesized. This suggests that the photocleavage activity is dependent on the structure of the dyes and thus would allow the development for a dyes system with ideal photo physical properties to be further developed as PDT agents. Future Direction allow for modifications that will facilitate tumor cell selectivity and specific subcellular localization of the PDT agents that could be individually fine-tuned for an exact therapeutic requirement.

3.8 Experimental for Chapter 2: Pentamethine Dyes

Synthesis of N-([1,1'-biphenyl]-2-yl)acetamide 32

64.0 mmol (10.3 g) of [1,1'-biphenyl]-2-amine was added to 60 mL of acetic anhydride (Ac₂O) (10 eq) and stirred for 2 h at room temperature. Upon cooling the solution to room temperature, the solution was poured over in ice bath to precipitate out 32 as an egg shade/off white solid.
The product was collected under vaco. and washed with deionized water. The solid was then dried under vaco. and was used as in latter steps. The reaction was monitored via thin layer chromatography (TLC) using dichloromethane (DCM) as the eluting solution. 13.0 g Yield 96 %

Synthesis of 6-methylphenanthridine 33

62.0 mmol (13 g) was refluxed in 45 mL of phosphoryl chloride (POCl₃) (7 eq) for 3.75 h under nitrogen. Upon cooling the solution to room temperature, the solution was poured over in ice bath to precipitate the salt of 33 as an off-white solid. The solid was collected under vaco. and was washed with deionized water. The solid was then added to a concentrated solution of sodium carbonate (Na₂CO₃) and extracted twice with 50 mL DCM. The organic layers were then concentrated using a rotary evaporator to afford 33 as a dull orange solid.

Yield 76 %, 9 g; ¹H NMR (400 MHz, DMSO-d₆): δ ppm 3.31 (s, 3H), 7.97-8.09 (m, 3H), 8.22 (d, J = 8.0 Hz, 1H), 8.33 (t, J = 13.2Hz, 1H), 8.71 (d, J = 8.4 Hz, 1H), 9.03 (d, J = 8.0 Hz, 1H), 9.09 (d, J = 8.4 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): 19.09, 123.44, 123.73

General Synthetic Procedure for the phenanthridinium salts

10 mmol (2.0 g) of 15 was heated in the appropriate alkyl halide for 3 days in a sealed tube. The reaction was monitored via TLC using DCM as the eluting solution. Upon cooling to room temperature, the solution was added diethyl ether to precipitate out the salt. The solid was the collected under vaco. and washed with diethyl ether and acetone. The salt 4c was used without further purification in subsequent reactions.

5,6-dimethylphenanthridin-5-ium iodide 34a; yellow solid, Yield 72 %; ¹H NMR (400 MHz, DMSO-d₆): δ ppm 3.58 (s, 3H), 4.58 (s, 3H), 8.04-8.13 (m, 3H), 8.25 (t, J = 8.0 Hz, 1H), 8.64 (d, J = 8.4 Hz, 1H), 8.92 (d, J = 8.4 Hz, 1H), 9.15 (d, J = 8.4 Hz, 2H); ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 19.17, 40.87, 119.60, 122.91, 123.93, 124.02, 129.12, 129.73, 130.19, 131.23, 132.65, 134.52, 136.34, 165.44 m.p. > 260 °C

5-ethyl-6-methylphenanthridin-5-ium iodide 34b; yellow solid, Yield 62 %; ¹H NMR (400 MHz, CDCl₃-d): δ ppm 1.63 (t, J=7.08 Hz, 3 H) 3.47 (s, 3 H) 5.13 (d, J=7.17 Hz, 2 H) 8.01 - 8.15
(m, 3 H) 8.34 (t, J=7.77 Hz, 1 H) 8.64 (d, J=8.70 Hz, 1 H) 8.91 (d, J=8.19 Hz, 1 H) 9.14 (t, J=8.88 Hz, 2 H)

**34c 5-butyl-6-methylphenanthridin-5-ium iodide** yellow solid, Yield 68%, $^1$H NMR (400 MHz, CHLOROFORM-$d$) d ppm 1.13 (t, J=7.34 Hz, 3 H), 1.80 (d, J=7.34 Hz, 2 H), 2.05 - 2.28 (m, 2 H), 3.81 (s, 3 H), 5.33 (d, J=6.66 Hz, 2 H), 7.98 - 8.09 (m, 3 H), 8.25 (t, J=8.02 Hz, 1 H), 8.38 (d, J=9.05 Hz, 1 H), 8.77 (d, J=8.53 Hz, 1 H), 8.81 - 8.89 (m, 2 H)

**General Synthetic Procedure for 34a-e**

At a ratio of 2:1, 2 mmol of the appropriate salt 15a-c to the polymethine regent, ((E)-N-((E)-3-(phenylamino)allylidene)benzenaminium chloride were dissolved in 5 mL of Ac$_2$O. To this solution, 2 mol. eq. of triethylamine (TEA) was added. The reaction mixture was heated to 75 °C. The reaction was monitored via UV-Vis. After the reaction was completed, ethyl ether was added to afford the dyes.

The dyes were purified via column chromatography solvent ratio of 1:25 methanol to methylene chloride.

**5-methyl-6-((1E,3E,5E)-3-(5-methylphenanthridin-6(5H)-ylidene)penta-1,3-dien-1-yl)phenanthridin-5-ium iodide 35a;** dark purple solid, Yield 25 % $^1$H NMR (400 MHz, DMSO-$d_6$): δ ppm 4.10 (s, 6H), 6.79 (t, J = 8.8 Hz, 1H), 6.91 (d, J = 13.6, 2H), 7.76-7.62 (m, 4H), 7.77 (q, J = 6.4, 4H). 7.88 (d, J = 9.2 Hz, 2H), 7.94 (t, J = 8.0 Hz, 2H), 8.36 (d, J = 7.6 Hz, 2H) 8.61-8.68 (m, 4H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ ppm 41.55, 108.96, 117.11, 121.70, 122.54, 123.07, 125.65, 126.20, 128.00, 128.62, 130.30, 131.14, 132.50, 137.65, 147.99, 153.12; $\lambda_{\text{max in MeOH}}$ = 714 nm; m.p. = 176-178 °C

**6-((1E,3Z,5E)-3-chloro-5-(5-methylphenanthridin-6(5H)-ylidene)penta-1,3-dien-1-yl)-5-methylphenanthridin-5-ium iodide 35b;** Yield 46 % $^1$H NMR (DMSO-d6 ,400 MHz): δ ppm 4.16 (s, 6 H) 6.90 (d, J = 13.39 Hz, 2 H) 7.64 (t, J = 7.71 Hz, 2 H) 7.74 - 7.85 (m, 6 H) 7.93 - 8.04 (m, 4 H) 8.36 (d, J = 8.08 Hz, 2 H) 8.69 (d, J = 8.34 Hz, 2 H) 8.74 (d, J = 7.83 Hz, 2 H) ;
13C NMR (DMSO-d6, 100 MHz): δ ppm 42.25, 105.53, 118.02, 122.23, 122.37, 123.15, 123.7, 125.79, 126.23, 128.77, 129.46, 131.00, 131.84, 133.55, 137.65, 143.98, 155.15

5-ethyl-6-((1E,3E,5E)-5-(5-ethylphenanthridin-6(5H)-ylidene)pent-1,3-dien-1-yl)phenanthridin-5-ium iodide 53c; dark green solid, 19 %; 1H NMR (400 MHz, DMSO-d6): δ ppm 1.47 (t, J = 6.8 Hz, 6H), 4.61 (q, J = 7.2 Hz, 4H), 6.74 (d, J = 14.0 Hz, 2H), 6.84 (t, J = 13.2 Hz, 1H), 7.47-7.57 (m, 4H), 7.75 (q, J = 7.2 Hz, 4H) 7.92-7.97 (m, 4H), 8.23 (d, J = 8.0 Hz, 2H), 8.64 (t, J = 7.2 Hz, 4H); 13C NMR (100 MHz, DMSO-d6): δ ppm 12.29, 45.67, 108.98, 116.89, 122.03, 122.38, 123.44, 124.49, 125.65, 125.89, 128.44, 129.73, 130.48, 132.66, 135.68, 147.99, 152.68, λmax in MeOH = 712 nm; mp = 185 °C decomp.

6-((1E,3Z)-3-chloro-((E)-5-ethylphenanthridin-6(5H)-ylidene)penta-1,3-dien-1-yl)-5-ethylphenanthridin-5-ium iodide 35d 1H NMR (400 MHz, DMSO-d6): δ ppm 1.54 (br. s., 7 H), 4.68 (d, J=5.56 Hz, 4 H), 6.74 (d, J=13.64 Hz, 2 H), 7.62 (br. s., 2 H), 7.71 - 7.88 (m, 7 H), 8.02 (t, J=7.96 Hz, 4 H), 8.28 (d, J=8.08 Hz, 2 H), 8.72 (br. s., 4 H); 13C NMR (100 MHz, DMSO-d6): δ ppm 12.99, 46.94, 105.77, 118.13, 123.41, 124.51, 131.31, 131.71, 132.93, 134.20, 136.12, 144.99,

6-((1E,3Z)-3-bromo-((E)-5-ethylphenanthridin-6(5H)-ylidene)penta-1,3-dien-1-yl)-5-ethylphenanthridin-5-ium iodide 35e 1H NMR (400 MHz, DMSO-d6): δ ppm 1.55 (t, J = 6.82 Hz, 6H) 4.66 (d, J = 7.07 Hz, 4 H) 6.72 (d, J = 13.14 Hz, 2 H) 7.62 (br. s., 2 H) 7.71 - 7.84 (m, 6 H) 7.96 - 8.05 (m, 4 H) 8.26 (d, J=8.08 Hz, 2 H) 8.71 (t, J = 6.82 Hz, 4 H)

5-butyl-6-((1E,3E,5E)-5-(5-butylphenanthridin-6(5H)-ylidene)penta-1,3-dien-1-yl)phenanthridin-5-ium iodide 35f; dark purple solid, Yield 29 % 1H NMR (400 MHz, DMSO-d6): δ ppm 0.94 (t, J = 7.2 Hz, 6H), 1.43 (m, J = 8 Hz, 4H), 1.81 (m, J = 6 Hz, 4H) 4.54 (t, J = 7.6 Hz, 4H), 6.75 (d, J = 13.2 Hz, 2H), 6.82 (t, J = 12.8, 1H), 7.46 (t, J = 12.8, 2H), 7.45 (t, J = 7.6, 2H), 7.74 (q, J = 7.6 Hz, 4H), 7.89-7.96 (m, 4H), 8.22 (d, J = 8.0 Hz, 2H), 8.62 (t, J = 8.0 Hz, 4H); 13C NMR (100 MHz, DMSO-d6): δ ppm 13.06, 18.73, 28.57, 50.81, 109.55, 117.10, 117.19,
122.19, 122.39, 123.45, 124.53, 125.81, 125.95, 128.33, 128.49, 129.25, 129.37, 130.37, 131.59, 132.62, 135.99, 147.43, 153.17; $\lambda_{\text{max}} = 710$ nm; mp = $187 \, ^\circ\text{C}$

1-methyl-4-((1E,3E)-5-((Z)-1-methylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)quinolin-1-ium iodide 42a
$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 3.97 (s, 6 H) 6.65 (t, $J=12.25$ Hz, 1 H) 6.99 (d, $J=13.39$ Hz, 2 H) 7.32 (d, $J=7.33$ Hz, 2 H) 7.59 (t, $J=7.07$ Hz, 2 H) 7.78 - 7.89 (m, 4 H) 7.95 (t, $J=13.01$ Hz, 2 H) 8.09 (d, $J=7.07$ Hz, 2 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 41.96, 108.27, 110.97, 117.78, 119.40, 124.70, 125.27, 126.44, 129.11, 133.00, 139.42, 141.95, 147.44

4-((1E,3E)-5-((Z)-1-methylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)-1-methylquinolin-1-ium iodide 42b
$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 4.05 (s, 6 H) 6.94 (d, $J=13.14$ Hz, 2 H) 7.41 (d, $J=7.33$ Hz, 2 H) 7.67 (t, $J=5.81$ Hz, 2 H) 7.86 - 7.97 (m, 4 H) 8.14 (d, $J=12.88$ Hz, 2 H) 8.30 (d, $J=7.07$ Hz, 2 H) 8.41 (d, $J=8.34$ Hz, 2 H)

1-ethyl-4-((1E,3E)-5-((Z)-1-ethylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)quinolin-1-ium iodide 42d
$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 1.38 (t, $J = 7.07$ Hz, 6 H) 4.40 (q, $J = 6.99$ Hz, 4 H) 6.63 (t, $J = 12.38$ Hz, 1 H) 6.93 (d, $J = 13.39$ Hz, 2 H) 7.31 (d, $J = 7.07$ Hz, 2 H) 7.53 (t, $J = 7.45$ Hz, 2 H) 7.75-7.88 (m, 4 H) 7.95 (t, $J = 12.88$ Hz, 2 H) 8.09 (d, $J = 7.07$ Hz, 2 H) 8.36 (d, $J = 8.34$ Hz, 2 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ ppm 14.91, 48.91, 108.73, 111.02, 117.47, 124.90, 125.57, 126.22, 133.01, 138.16, 140.79, 147.39

4-((1E,3Z)-3-chloro-5-((Z)-1-ethylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)-1-ethylquinolin-1-ium iodide 42e
$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 1.44 (t, $J = 6.57$ Hz, 6 H) 4.51 (d, $J = 6.82$ Hz, 4 H) 6.93 (d, $J = 12.88$ Hz, 2 H) 7.65 (t, $J = 7.33$ Hz, 2 H) 7.84 - 7.93
(m, 2 H) 7.99 (d, J = 8.59 Hz, 2 H) 8.16 (d, J = 13.14 Hz, 2 H) 8.34 (d, J = 6.82 Hz, 2 H) 8.40 (d, J = 8.08 Hz, 2 H)

4-((1E,3Z)-3-chloro-5-((Z)-1-butylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)-1-butylquinolin-1-ium iodide 42f

$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 0.93 (br. s., 6 H) 1.37 (br. s., 4 H) 1.80 (br. s., 4 H) 4.47 (br. s., 5 H) 6.95 (d, J = 11.62 Hz, 2 H) 7.44 (br. s., 2 H) 7.66 (br. s., 2 H) 7.91 (br. s., 2 H) 7.99 (br. s., 2 H) 8.15 (d, J = 12.88 Hz, 2 H) 8.32 (br. s., 2 H) 8.42 (br. s., 3 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ ppm 14.01, 19.75, 31.27, 54.02, 106.68, 109.33, 118.14, 122.71, 125.00, 125.53, 127.02, 133.52, 138.30, 141.50, 142.47, 148.60

4-((1E,3Z)-3-bromo-5-((Z)-1-butylquinolin-4(1H)-ylidene)penta-1,3-dien-1-yl)-1-butylquinolin-1-ium iodide 42g

$^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 0.93 (br. s., 6 H) 1.38 (d, J=5.81 Hz, 4 H) 1.80 (br. s., 4 H) 4.48 (br. s., 4 H) 6.92 (d, J = 12.38 Hz, 2 H) 7.46 (br. s., 2 H) 7.67 (br. s., 2 H) 7.92 (br. s., 2 H) 8.00 (br. s., 2 H) 8.25 (d, J = 12.88 Hz, 2 H) 8.36 (br. s., 4 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ ppm 14.01, 19.75, 31.28, 54.02, 106.68, 109.33, 118.13, 122.71, 125.00, 125.53, 127.02, 133.52, 138.30, 141.50, 142.47, 148.60
4 Synthesis and Evaluation of Novel Near-Infrared Phenoxazinium Dyes as Potential Contrast Agents for Imaging Insulinoma

This chapter includes the synthesis of near-infrared phenoxazinium dyes as potential as contrast agents for insulinoma.

4.1 Summary of Insulinoma, a Pancreatic Cancer

Figure 37 The pancreas

The pancreas is both an endocrine gland and part of the digestive system, thus containing two different types of glands: exocrine and endocrine, as shown in Figure 37. The endocrine pancreas gland contains four types of cells, with each responsible for the hormone secretion of glucagon, pancreatic polypeptide, somatostatin, and insulin respectively. The exocrine pancreas gland contains two types of cells, which release pancreatic juices with digestive enzymes to aid in digestion and absorption of nutrients. Due to the many functional cell types of the pancreas, cancer of the pancreas can have two manifestations, functioning and nonfunctioning. In 2012 alone, pancreatic cancers caused more than 330,000 deaths globally and an estimated 48,000 new cases are expected to occur in the US in 2015. Nonfunctioning tumors may cause obstructive symptoms of the biliary tract or duodenum, bleeding into the GI tract, or abdominal masses. Functioning tumors hypersecrete a particular hormone, causing various syndromes. Neuroendocrine tumors of the pancreas and duodenum include gastrinomas, vasoactive intesti-
nal peptideoma (VIPomas), somatostatinomas (SST), and insulinomas, with the latter accounting for 17% of all cases. Insulinoma, the most frequent form of pancreatic neuroendocrine tumors of β-cell origin and although only 10% of insulinomas are malignant, all cause hyperinsulinemic hypoglycemia (low blood glucose caused by excessive insulin). Diagnosis of insulinoma is defined clinical parameters derived from the Whipple triad, hypoglycemia symptoms in the presence of low plasma glucose with clinical relief on glucose administration. As almost all (97%) insulinoma are located in the pancreas, few methods provide specific contrast intraoperatively as only 80% of insulinoma are single with localization of tumors less than 2 cm remaining difficult using a single imaging technique. During surgery, it is very important to distinguish between cancerous and healthy tissues in real-time with high sensitivity to avoid such injury to other organs. If tumors not effectively identified, incomplete or blind resection and/or the need for re-exploration can result in clinical syndromes leading to multiple endocrine neoplasia, in which tumors or hyperplasia affects additional endocrine glands.

With the current limit of detection, diagnosis of the cancer has usually either had spread throughout the body or has fully developed thus rendering treatment ineffective, with surgical removal of the tumor is the treatment of choice for insulinoma. Furthermore, there is a need to develop better imagine techniques to assist in identify the cancer at various stages and during surgery. An ideal method for detection for insulinoma cancer would be noninvasive with minimal side effects to the patient and a low limit of detection which would for allow early detection.

4.2 Rational of NIR Fluorophore

Medical Imaging is limited to the optical window which is defined as the range of wavelengths between NIR 650 - 900 nm where light has its maximum depth of penetration in tissue and minimal interference from native biological tissues and molecules, as shown in Figure 38.
Figure 38 Optical window on in vivo bio imaging

Near-infrared (NIR) fluorescence imaging is a promising technique to facilitate intraoperative, real time, visual identification of tumors\textsuperscript{76-78}. Although several near-infrared (NIR) fluorophores have shown promise in preclinical model systems, there are no tumor-specific contrast agents presently available for clinical use. In fact, the only 2 NIR fluorescent contrast agents that are available clinically and approved, the 700 nm fluorophore methylene blue (MB) and 800 nm fluorophore indocyanine green (ICG), as shown in Figure 39. Furthermore MB and ICG have been clinically used for decade yet both agents are classified as non-specific with respect to targeting.

![Figure 39 FDA approved NIR fluorescent probes](image)

The combination of a NIR fluorescent contrast agent and appropriate imaging system could help the surgeon localize the desired disease, to define the boundaries of vital organs to find small occult metastases within the surgical field\textsuperscript{79}. The ideal fluorophore for the optical imaging would have properties that allows aqueous solubility, large Stokes shifts, large extinction coefficient ($\varepsilon>50,000$ M$^{-1}$ mol), long emission wavelength ($\lambda>680$ nm), and high quantum yield ($\Phi$). The
long wavelength would allow for less light scattering and deeper penetration since the pancreas is located behind the stomach, shown in Figure 40.

Figure 40 Anatomy of the pancreas, taken without permission.

Recently MB has shown uptake in the pancreas and localization in cancerous tissues and although MB does provide contrast between the pancreas and surrounding organs, it is not an ideal NIR contrast agent because of low signal intensity, nonspecific uptake, and short retention time in the pancreas. Thus the synthesized fluorophore would utilize a three ring system as a base, as shown in Figure 41; when X is a nitrogen, oxygen, or sulfur the molecule would be phenazine, phenoxazine, and phenothiazine respectively with the aminos acting as a donor/acceptor group for tailoring the absorbance and emission bands.

Figure 41 Design of compact NIR contrast agent.

From the literature, there a trend in which there is an increase in both absorbance and fluorescence maxima with the heteroatom increasing down the same group. Phenothiazine (X = S) based dyes, such as methylene blue (MB) provides both absorbance and fluorescence within the NIR window (660-690 nm) but, are well studied for their production of $^1\text{O}_2$ which limits it use an imaging agent. Phenazine (X = N) based dyes have both absorbance and fluores-
cence bands within the yellow region (540-580 nm), thus having too short of wavelength maxi-
ma that would allow good contrast and not in the NIR window. Phenoxazine (\( X = O \)) based
dyes have both absorbance and fluorescence bands within the blue region (620-670 nm), mak-
ing it a suitable scaffold to explore as possible imaging agents. Although Nile blue derivatives
(benz[c]phenoxazine) provide additional conjugation that shift both fluorescence band, the lipo-
philicity of the dyes increases with organic content (i.e. alkyl, aryl,) limiting its solubility in a po-
lar media such as water. The donor groups on the nitrogen would shift the phenoxazine dye to
longer wavelengths and Increase the stoke shifts would further minimize interference from the
excitation source and minimize background interference. Furthermore, through our collabora-
tors at Beth Israel Deaconess Medical Center and Harvard Medical School, a preliminary of com-
cercial available oxazine dyes were evaluated but synthesis of these dye have not been reported.

4.3 Synthesis of Symmetrical and Unsymmetrical Phenoxazinium Dyes
Although Nile Blue (benz[a]phenoxazinium) derivatives with secondary amine have been re-
ported, the literature lacks of phenoxazine dyes with secondary amines is not reported. The
synthesis of phenoxazinium 49 and 50, as shown in Equation 9 is prepared from the condensa-
tion of a nitroso anisole 44 with aminophenol derivatives 47 or 48 in acidic medium under reflux.

\[
\begin{align*}
\text{Equation 5 Synthesis of phenoxazinium dye} \\
\text{The first half of the dye begins with the formation of the nitroso compounds 43, as shown in}
\end{align*}
\]

Scheme 9 from \( m \)-anisole.
The animo of the $m$-anisole was alkylated in basic conditions with the appropriate alkyl halide at. Under these basic conditions, mono-, di-, and tri alkylation of the amine occurred as shown by TLC. As such, flash column chromatography was used to isolate the di- and mono-alkylated anisoles 43a-b. After isolation, nitrosylation of the anisole was achieved with a solution of sodium nitrate ($\text{NaNO}_2$) and hydrochloric acid in water. When the reaction was completed, neutralizing the reaction mixture precipitated the dialkylated anisole nitroso compounds out of aqueous solution. In contrast, the monoalkylated anisole was extracted with ethyl acetate from the aqueous reaction due to it forming an oil upon neutralizing the reaction mixture.

To alter the hydrophobicity of the overall dye, the second half of the dye was obtained from either 4-methyl-3nitro-2-phenol 45 or m-phenol, shown in Scheme 10.

Scheme 9 Synthesis of Alkylated 4-Nitroso-3-aminoanisole

Scheme 10 Synthesis of Alkylated Aminophenol

The nitro compound 45 was reduced using Pd/C in methanol with ammonia formate ($\text{NH}_4\cdot\text{HCO}_2$). The aminophenols were alkylated in basic conditions with the appropriate alkyl
halide. Under these basic conditions, mono-, di-, and tri alkylation of the amine occurred as shown by TLC. As such, flash column chromatography was used to isolate the di- and mono-alkylated aminopheneols 47-48a-b.

Scheme 11 Synthesis of Symmetrical and Unsymmetrical Phenoazinium Dyes

After isolation of the respective halves, a mixture of the phenol 47 and nitroso compound 44 with the appropriate acid were slowly heated to reflux, with color change in the solution occurring with the first four hours of the initial heating and mixing. The reaction was monitored via UV-Vis until there was no change in its absorption spectra by following the decrease in absorption of the starting materials and increase in absorption of the dye 49/50. After heating, the reaction mixture was cooled and concentrated under reduced pressure. The dark residue was then dissolved in a mixture of 1:1 methanol: acetone and was loaded on the column. The dye eluted using a gradient mobile phase composed of acetone and methanol. Chloroform was omitted as a mobile phase due to acetone providing better solubility of concentrated residue. After concentrating the dark blue fractions from the column, they were concentrated under reduced pressure and with minimal methanol (approx. 2 ml) present in the flask a 1:1 diethyl ether: ethyl acetate solution was added to precipitate the dyes as dark blue solids. The dye was collected under re-
duced pressure and recrystallized from acetone in ethyl acetate to achieve a high purity sample for NMR analysis.

### 4.3.1.1 Computational methods

The Calculated LUMO and HOMO orbitals were obtained using a restricted hybrid HF-DFT SCF calculation performed using Pulay DIIS + Geometric Direct Minimization and 6-31G* basis set, B3LYP Method via Spartan or Gassuioin(03).

### 4.4 Characterization

Compound 49a was chosen as a representative for complete chemical characterization and analysis via NMR ($^{13}$C and $^1$H). The $^1$H spectra of 49a are shown in Figure 43 in CD$_3$OD-d$_4$. Analyzing the dye structure of 49a, the symmetrical nature of the dyes allows the dye to have four signals in the $^1$H spectra, as shown in Figure 43.

![Figure 42 $^1$H NMR spectrum of 49a in CD$_3$OD-d$_4$ at 25°C](image)

The spectra of 49a show three peaks in the aromatic region consisting of two doublets ($J = 7.10$ Hz) and a singlet, which agrees with both the integration and expected splitting pattern of the dye molecule. Furthermore, a singlet appears in the aliphatic region, which is expected from the di-methyl of the amines. The $^{13}$C spectra of 49a are shown in Figure 44 in CD$_3$OD-d$_4$. The $^{13}$C
spectrum of $49a$ shows all seven expected signals from the dye molecule. In the aromatic region, the two peaks furthest downfield would correspond to the carbons connected directly to the heteroatom of the tricyclic ring system.

![Figure 43 NMR spectrum of $49a$ in CD$_2$OD-d$_4$ at 25°C](image)

The carbon connected to the alkylated amines would appear further up field when compared to the other aromatic peaks due to the electron donating nature of the amino group. Also a single peak appears in the aliphatic region, which is expected from the dimethyl of the amine.

When the symmetry of the dye is altered, a more complex spectra is observed due to more signals as shown in Figure 45.

![Figure 44 Top: $^1$H NMR spectrum of $49a$ in CD$_2$OD-d$_4$ at 25°C; Bottom: $^1$H NMR spectrum of $49c$ in bottom CD$_3$SO-d$_6$ at 25°C](image)
4.5 Results and Discussion

Scheme 12 Synthesis of Symmetrical and Unsymmetrical Phenoxazinium Dyes

For the synthesis of 3,7-bis(dialkyamino)phenoxaziniums, the use of starting material with secondary amines, specify the monoalkylated-nitrosoanisoles, required perchloric acid for the reaction to proceed as shown in Scheme 12. The physical properties for the dyes 49a-d,50 where calculated using ChemAxon(Budapest, Hungry) are found in Table 5.

<table>
<thead>
<tr>
<th>Dye</th>
<th>logD</th>
<th>Van der waal volume $\AA^3$</th>
<th>Polar Surface Area (2D)</th>
</tr>
</thead>
<tbody>
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<td>2.61</td>
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<td>49a</td>
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<td>310.02</td>
<td>32.51</td>
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<tr>
<td>49b</td>
<td>4.30</td>
<td>330.89</td>
<td>32.51</td>
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<td>371.90</td>
<td>41.30</td>
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<tr>
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<td>309.30</td>
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<tr>
<td>50b</td>
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<td>50.09</td>
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Table 5 The physical properties for the dyes 49a-e,50 where calculated using ChemAxon(Budapest, Hungry)

Both MB and dye 49a have similar physical properties but observe different photo-physical properties as shown in Figure 46.
Optical Properties of Phenoxazinium Dyes

The longer fluorescence lifetime and higher quantum yield would provide sharper contrast when compared to MB. Through our collaborators at Beth Israel Deaconess Medical Center and Harvard Medical School, the biodistribution for Dye 49a is shown in Figure 47. The imaging data shows different stages of the insulomia cancer as characterized by the size of the tumor as shown in Figure 47. Pancreatic cancer has three stages: Hyperplasia, Angiogenic, and Insulinoma in which they differ in vascularization, the formation of blood vessels and capillaries around the pancreatic islet. Stage one, Hyperplasia, has normal pancreatic islet cell vasculature. Furthermore, the contrast as a ratio of Insulinoma to pancreas of dye 49a when compared to MB. Specify of the dye towards Insulinoma was shown on Figure 48 which shows MRI tracking and Co-registration of dye 49a.
Six novel symmetrical and unsymmetrical Phenoxazinium dyes were synthesized in moderate yield (7-50 %). The dye 49a showed better selectivity for Insulinoma when compared to MB.
Enabling this technology with recent advancements in real time intraoperative imaging systems would allow for real-time localization of Insulinoma for the surgeon.

4.7 Experimental

Synthesis of 3-methoxy-N,N-dialkylaniline 43a and 3-methoxy-N-alkylalaniline 43b

A mixture of m-anisole (49 mmol), potassium carbonate (3 mol. eq.) in DMF (40 ml) was stirred at RT for 0.5 h. After stirring, iodomethane (2 mol. eq.) was added drop wise. After the addition, the mixture was heated to 50°C for 18 h. After the mixture cooled, DI water was added and compounds 43a and 43b were extracted three times with 75 mL with ethyl acetate. The fractions were then concentrated and loaded on silica for purification via column chromatography using a gradient solvent system of hexanes and ethyl acetate.

3-methoxy-N,N-dimethylaniline 43a Yield 51 %; \(^1\)H NMR (400 MHz, CDCl\(_3\)-d): \(\delta\) ppm 3.02 (s, 6 H) 3.89 (s, 3 H) 6.35 - 6.42 (m, 2 H) 6.44 - 6.50 (m, 1 H) 7.25 (t, \(J = 7.96\) Hz, 1 H)

3-methoxy-N-methylaniline 43b Yield 28 %; \(^1\)H NMR (400 MHz, CDCl\(_3\)-d) \(\delta\) ppm 2.87 (s, 3 H) 3.84 (s, 3 H) 6.17 - 6.25 (m, 1 H) 6.30 (dd, \(J = 8.00\), 0.90 Hz, 1 H) 6.35 (dd, \(J = 8.10\), 1.10 Hz, 1 H) 7.16 (t, \(J = 8.08\) Hz, 1 H)

Synthesis of 3-methoxy-N,N-dimethyl-4-nitrosoaniline 44a

A mixture of compound 43a (26 mmol) and HCl (1.2 mol. eq.) in H\(_2\)O (30 ml) was stirred in an ice bath. NaNO\(_2\) (1 mol. eq.) was slowly added to the mixture dropwise for 60 min and then the resulting mixture was stirred in ice bath for another 1 h. After addition of K\(_2\)CO\(_3\) powder to basicify the above solution until pH \(\sim 9\), the mixture was filtrated and the green mass was washed with water. The mass was ultrasonicated in Et\(_2\)O and filtrated to give nitroso compound 44a. Yield 81 % \(^1\)H NMR (400 MHz, CDCl\(_3\)-d) \(\delta\) ppm 3.15 (s, 6 H) 4.14 (s, 3 H) 6.01 - 6.17 (m, 2 H) 6.65 (d, \(J = 8.34\) Hz, 1 H): \(^{13}\)C NMR (100 MHz, CDCl\(_3\)-d) \(\delta\) ppm 40.61, 56.16, 92.28, 104.18, 156.10, 157.86

Synthesis of 3-methoxy-N-methyl-4-nitrosoaniline 44b
A mixture of compound 43a (0.9 g, 6.56 mmol) and HCl (1.2 mol. eq.) in H₂O (30 ml) was stirred in an ice bath. NaNO₂ (1 mol. eq.) was slowly added to the mixture dropwise for 60 min and then the resulting mixture was stirred in ice bath for another 1 h. After addition of K₂CO₃ powder to basicify the above solution until pH ~9, the mixture was added to 300 ml of DI water and washed 3x 75 mL of ethyl acetate. The organic layers were dried of magnesium sulfate and concentrated to give the nitroso compound 44b as oil. (1 g, 6.02 mmol, 81 % yield)

1H NMR (400 MHz, CDCl₃-d) δ ppm 3.15 (s, 6 H) 4.14 (s, 3 H) 6.01-6.17 (m, 2 H) 6.65 (d, J = 8.34 Hz, 1 H): 13C NMR (100 MHz, CDCl₃-d) δ ppm 40.61, 56.16, 92.28, 104.18, 156.10, 157.86

Synthesis of 3-amino-4-methylphenol 46

Ammonium formate (15 g, 238 mmol) was dissolved in 50 ml MeOH and added to 50 ml of MeOH containing 3-amino-4-methylphenol (10.5 g, 68 mmol). To the solution, approx. 1 g of Pd/C was added and heated to 60 °C. The Rxn was monitored via TLC (4:1 HEX:EYLAc) showed completion after 18 h. The reaction mixture filtered via vaco and the mother liquid was concentrated. The residue was then washed with 400 ml of DI water and extracted with 3x 75 ml of EtOAc. Yield 89 % 1H NMR (400 MHz, CDCl₃-d): δ ppm 2.10 (s, 3 H) 6.10-6.26 (m, 6 H) 6.89 (d, J = 7.58 Hz, 1 H)

3-(N-methylamino)-4-methylphenol 47a Yield 42 % 1H NMR (400 MHz, CDCl₃-d): δ ppm 2.08 (s, 3 H), 2.85 (s, 3 H), 6.15 (s, 2H), 6.90 (d, J = 7.6 Hz, 1 H)

3-(dimethylamino)-4-methylphenol Yield 57 % 47b 1H NMR (400 MHz, CDCl₃-d): δ ppm 2.26 (s, 3 H), 2.69 (s, 6 H), 6.44 (dd, J = 6.0 Hz, J = 2.4 Hz, 1 H), 6.55 (d, J = 2.0 Hz, 1 H) 7.01 (d, J = 8.0 Hz, 1 H); 13C NMR (100 MHz, CDCl₃-d): δ ppm 17.71, 44.04, 105.90, 109.08, 123.72, 131.81, 153.69, 154.28

Synthesis of 3-(dimethylamino)phenol 48a and 3-(methylamino)phenol 48b

A mixture of compound m-aminophenol (93 mmol) and potassium carbonate (3 mol. eq.) in DMF (40 ml) was stirred at RT for 1h. After stirring, iodomethane (2 mol. eq.) was added dropwise. After the complete addition, the mixture was heated to 40 °C for 3 h. After the mixture
cooled, DI water was added and compounds were extracted three times with 100 ml with ethyl acetate. The fractions were concentrated and was purified via column chromatography using a gradient solvent system of hexanes and with ethyl acetate.

3-(dimethylamino)phenol 48a Yield 38% 1H NMR (400 MHz, CDCl3-d) : δ ppm 2.91 (s, 6 H) 6.21 - 6.30 (m, 2 H) 6.36 - 6.44 (m, 1 H) 7.13 (t, J = 1.00 Hz, 1 H)

3-(methylamino)phenol 48b Yield 48% 1H NMR (400 MHz, CDCl3-d) : δ ppm 2.80 (s, 3 H) 6.07 - 6.14 (m, 1 H) 6.18 - 6.27 (m, 2 H) 7.04 (t, J = 7.96 Hz, 1 H); 13C NMR (100 MHz, CDCl3-d) : δ ppm 30.91, 99.92, 104.96, 105.85, 130.22, 150.70, 156.85

General Procedure for Syntheisis of Symmetrical and Unsymmetrical Phenoxazinium Dyes

A mixture of compounds 44 with 2 mol eq of perchloric acid in i-PrOH (10 ml) was stirred at 30°C. A solution of compound 48 in 90% i-PrOH (10 ml) was added dropwise to the above mixture during 45 min. The reaction was monitored via UV-Vis. The dark blue solution was evaporated and the residue was purified by column chromatography with silica gel, eluting by CHCl3/Acetone from 10:1 to 1:1 (v/v) and the dark blue solution was evaporated. To a solution of the residue EtOH or MeOH (2 mL), was added AcOEt (20 mL). After ultrasonication for 10 min, the mixture was filtrated. The powder was washed by AcOEt and Et2O then dried in vacuum.

N-(7-(dimethylamino)-3H-phenoazin-3-ylidene)-N-methylmethanaminium chloride 49a

Yield 35% 1H NMR (400 MHz, METHANOL-d4) δ ppm 3.42 (s, 12 H), 6.90 (s, 2 H), 7.37 (d, J = 7.07 Hz, 2 H), 7.72 (br. s., 2 H); 13C NMR (100 MHz, METHANOL-d4) δ ppm 40.40, 96.12, 117.18, 133.76, 148.92, 157.90
(Z)-N-(7-(dimethylamino)-2-methyl-3H-phenoxazin-3-ylidene)ethanaminium perchlorate

49b Yield 7 % ¹H NMR (400 MHz, DMSO-d₆) δ ppm 2.26 (s, 3 H) 3.10 (br. s., 3 H) 3.33 (s, 6 H) 6.74 (s, 1 H) 6.84 (d, J=1.77 Hz, 1 H) 7.32 (dd, J=9.47, 1.89 Hz, 1 H) 7.56 (s, 1 H) 7.73 (d, J=9.35 Hz, 1 H)¹³C NMR (100 MHz, DMSO-d₆): δ ppm 31.15, 31.49, 41.52, 94.32, 96.40, 117.41, 129.60, 132.35, 133.13, 133.55, 134.76, 148.38, 149.00, 156.89, 158.52

N-methyl-N-(2-methyl-7-(methylamino)-3H-phenoxazin-3-ylidene)ethanaminium perchlorate 49c Yield 38 % ¹H NMR (400 MHz, DMSO-d₆) δ ppm 2.52 (s, 3 H) 3.11 (d, J=4.29 Hz, 3 H) 3.30 (s, 6 H) 6.82 (br. s., 1 H) 6.94 (s, 1 H) 7.13 - 7.26 (m, 1 H) 7.67 (br. s., 2 H) ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 22.24, 31.16, 44.33, 94.31, 100.53, 122.50, 130.35, 133.39, 133.99, 135.06, 136.66, 146.73, 150.56, 159.86, 160.32

(Z)-N-(7-(dimethylamino)-2-methyl-3H-phenoxazin-3-ylidene)ethanaminium perchlorate

49d Yield 30% ¹H NMR (400 MHz, DMSO-d₆) δ ppm 1.27 (t, J=12.8, 4 H), 2.30 (s,3 H), 3.28 (s, 6 H), 3.50 (q, J=5.81 Hz, 2 H) 6.69 (br. s., 2 H) 7.21 (d, J=9.09 Hz, 1 H) 7.42 (br. s., 1 H) 7.59 (d, J=9.09 Hz, 1 H) ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 13.86, 17.42, 39.02, 39.06, 43.85, 43.99, 93.87, 95.96, 116.93, 129.33, 132.21, 132.64, 133.17, 134.19, 147.82, 148.52, 156.44, 157.25

(Z)-N-(7-(butylamino)-2,8-dimethyl-3H-phenoxazin-3-ylidene)ethanaminium perchlorate

50a Yield 43% ¹H NMR (400 MHz, Acetone) δ ppm 0.98 (t, J=7.33 Hz, 3 H) 1.39 (t, J=7.20 Hz, 4 H) 1.44 - 1.57 (m, 2 H) 1.79 (t, J=7.33 Hz, 2 H) 2.40 (br. s., 6 H) 3.61 - 3.77 (m, 4 H) 6.92 (s, 2 H) 7.64 (br. s., 2 H) ¹³C NMR (100 MHz, acetone-d₆): δ ppm 13.15, 13.19, 16.58, 30.44, 38.92, 39.06, 43.85, 43.99, 93.54, 93.59, 93.67, 93.72, 129.07, 129.12, 129.17, 132.40, 134.08, 134.13, 148.89, 157.05, 157.14, 157.23, 157.32
REFERENCES


6 APPENDICES

6.1 Appendix A

Chapter 1: $^1$H NMR, $^{13}$C NMR, and HRMS Spectra: $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer and high-resolution accurate mass spectra (HRMS) were obtained either at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro ESIQ-TOF mass spectrometer or utilizing a Waters Micromass LCT TOF ES+ Premier Mass Spectrometer.
Chemical Formula: $C_{30}H_{36}IN_2$
Molecular Weight: 550.53
Chemical Formula: C$_{30}$H$_{35}$N$_2$
Molecular Weight: 550.53
Emission of 27b in 90% Glycerol / 10 % Methonal (%v/%v) at 20 µM

Absorbance of 27b in Methanol

Absorbance in Methanol to determine $\varepsilon$ of 27b

Chemical Formula: $C_{30}H_{35}IN_2$
Molecular Weight: 550.53
Absorbance for 27b in 90% Glycerol/10% Methanol (%v/%v)

Absorbance for ε of 19 in 90% Glycerol/10% Methanol (%v/%v)

\[ y = 0.0262x - 0.0114 \]

\[ R^2 = 0.9994 \]

Chemical Formula: \( \text{C}_{30} \text{H}_{35} \text{N}_2 \)

Molecular Weight: 499.53
Chemical Formula: C\textsubscript{39}H\textsubscript{34}Cl\textsubscript{4}N\textsubscript{2}
Molecular Weight: 584.97

Chemical Formula: C\textsubscript{39}H\textsubscript{34}Cl\textsubscript{4}N\textsubscript{2}
Molecular Weight: 584.97
Emission of 27d in 90% Glycerol / 10% Methanol (%w/%v) at 20 µM

Chemical Formula: C_{30}H_{34}ClN_{2}
Molecular Weight: 584.97

Absorbance in Methanol to determine ε of 27d

y = 0.0236x + 0.0024
R² = 0.9967
Absorbance of 27d in 90% Glycerol/10% Methanol (%v/%v)

- 7 μM
- 12 μM
- 17 μM
- 19 μM
- 21 μM
- 23 μM

Absorbance for ε of 27d in 90% Glycerol/10% Methanol (%v/%v)

\[ y = 0.0273x + 0.078 \]

\[ R^2 = 0.9904 \]

Chemical Formula: \( C_{30}H_{34}ClN_2 \)
Molecular Weight: 584.97
Chemical Formula: C30H34ClN2+
Molecular Weight: 458.07

Cl

C

27d

LO-10-1
LO-10-1 509 (10.175) Cm (503:515)
Chemical Formula: C31H37IN2O

27c
Chemical Formula: C31H37IN2O

27c
Emission of 27c in 90% Glycerol / 10 % Methonal (%v/%v) at 20 µM

Absorbance of 27c in Methanol

Absorbance in Methanol to determine $\varepsilon$ of 27c

$y = 0.0236x + 0.0024$

$R^2 = 0.9967$

Chemical Formula: C31H37IN2O

27c
Absorbance for 27c in 90% Glycerol/10% Methanol (%v/%v)

Absorbance for $\varepsilon$ of 27c in 90% Glycerol/10% Methanol (%v/%v)

Chemical Formula: C31H37IN2O

27c
Chemical Formula: C28H30BrI2N2

27e
Fluorescence Intensity of 27e in 90% Glycerol / 10% Methonal (%v/%v) at 20 µM

Absorbance for ε of 27e in Methanol

Chemical Formula: C28H30BrI2N2

27e
Absorbance of 27e in 90% Glycerol/ 10% Methanol (%v/%v)

Absorbance for ε of 27e in 90% Glycerol/ 10% Methanol (%v/%v)

\[ y = 0.0304x - 0.0319 \]

\[ R^2 = 0.9979 \]
Chemical Formula: C$_{30}$H$_{34}$BrN$_2$
Molecular Weight: 629.42
27f

Chemical Formula: C$_{30}$H$_{34}$BrN$_{2}$
Molecular Weight: 629.42
Emission of 27f in 90% Glycerol / 10 % Methonal (%v/%v) at 20 μM

Fluorescence Intensity

λ (nm)

0 20 40 60 80 100 120

Absorbance of 27f in Methanol

Concentration (μM)

y = 0.0304x + 0.1004
R² = 0.9936

Chemical Formula: C_{30}H_{34}BrN_2
Molecular Weight: 629.42
Absorbance of 27f in 90% Glycerol/10% Methanol (%v/%v)

$y = 0.0286x - 0.0373$

$R^2 = 0.9982$

Chemical Formula: $C_{30}H_{34}BrIN_2$
Molecular Weight: 629.42
Chemical Formula: C35H36Br1N2
27g
Chemical Formula: C35H36BrN2

27g
Emission of 24 in 90% Glycercol / 10 % Methonal (%v/%v) at 20 µM

Absorbance for $\varepsilon$ of 24 in Methanol

Chemical Formula: C35H36BrI1N2
27g
Absorbance of 24 in 90% Glycerol/10% Methanol (%v/%v)

$y = 0.0399x - 0.0554$

$R^2 = 0.993$

Chemical Formula: C$_{33}$H$_{58}$BrN$_2$
Chemical Shift (ppm):

- 8.35
- 8.33
- 8.20
- 8.23
- 8.13
- 7.89
- 7.82
- 7.75
- 7.73
- 7.59
- 6.43
- 5.73
- 4.46
- 3.60

Chemical Formulas: C_nH_mN_p

Exact Mass: 431.25
Chemical Formula: C$_7$H$_7$N$_2$^+
Exact Mass: 431.25
y = 25337x + 0.1408
R² = 0.9966

Absorbance vs Concentration

Absorbance vs Wavelength (nm)
LO-5-DMSO-HNMR.001.001.1R.ESP

Chemical Formula: C_{17}H_{18}N_2^+

Exact Mass: 381.23
\[ y = 33343x + 0.0674 \]
\[ R^2 = 0.9945 \]
Chemical Formula: C_{36}H_{34}N_{3}O
Exact Mass: 535.18
Chemical Formula: C_{26}H_{22}N_{2}O_{2}^{+}
Exact Mass: 355.18
Absorbance vs Wavelength (nm)

$y = 32333x - 0.0078$

$R^2 = 0.9962$

Absorbance vs Concentration

Chemical Formula: C_{36}H_{27}N_2O^+
Exact Mass: 355.18
Chemical Formula: C24H23N2S+
Exact Mass: 371.16
Chemical Shift (ppm) 

Chemical Formula: C24H23N2S+ 
Exact Mass: 371.16
Absorbance vs. Wavelength:

Absorbance = 37660x + 0.1161

R² = 0.9955

Chemical Formula: C24H23N2S+
Exact Mass: 371.16
6.2 Appendix B

Chapter 2: $^1$H NMR, $^{13}$C NMR, and HRMS Spectra:

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer.
Chemical Formula: C_{14}H_{11}N
Molecular Weight: 193.25
Chemical Formula: C_{14}H_{11}N
Molecular Weight: 193.25
Chemical Formula: C_{16}H_{16}IN
Molecular Weight: 349.22
Chemical Formula: C_{35}H_{31}N_{2}
Molecular Weight: 606.55
Absorbance of **ESS-13** Methanol of Dye

- **Equation**: \( y = 0.0362x \)
- **R²**: 0.9997

![Graph of absorbance vs. concentration](chart.png)

**Chemical Structure of ESS-13**

- Chemical Formula: \( \text{C}_{35}\text{H}_{31}\text{IN}_{2} \)
- Molecular Weight: 606.55
- \( \lambda = 716 \text{ nm} \)
- \( \varepsilon = 36200 \)
Absorbance of ESS-13 in Methanol

Absorbance vs Wavelength (nm)
Chemical Formula: C_{35}H_{39}BrI\text{N}_2
Molecular Weight: 685.45
Chemical Formula: C_{35}H_{30}ClN_{2}
Molecular Weight: 640.99
Chemical Formula: C_{36}H_{30}ClN_2
Molecular Weight: 640.99
Chemical Shift (ppm):

Non-decoupled 1H NMR Spectra of C13.001.001.1r

Chemical Formula: C_{33}H_{27}I\text{N}_2
Molecular Weight: 578.50
Absorbance of ESS-37

\[ y = 0.0371x + 0.0105 \]

\[ R^2 = 0.999 \]

\[ \lambda = 725 \text{ nm} \]
\[ \varepsilon = 37,700 \]

Chemical Formula: \( \text{C}_{33}\text{H}_{27}\text{IN}_2 \)
Molecular Weight: 578.50
Chemical Formula: $C_{33}H_{26}ClIN_2$
Molecular Weight: 612.94

\[ \lambda = 718 \text{ nm} \quad \varepsilon = 41,700 \]
Chemical Formula: C_{33}H_{28}BrN_{2}
Molecular Weight: 657.39
Chemical Shift (ppm)

Chemical Formula: C_{33}H_{20}BrN_{2}
Molecular Weight: 657.39
Absorbance of ESS32

\[ y = 0.0408x \quad R^2 = 0.9974 \]

Chemical Formula: \( \text{C}_{33}\text{H}_{26}\text{BrI}\text{N}_2 \)
Molecular Weight: 657.39

\[ \lambda = 722 \text{ nm} \quad \varepsilon = 40,800 \]
Absorbance of ESS16

$y = 0.0399x - 0.0005$

$R^2 = 0.9993$

Chemical Formula: C$_{39}$H$_{39}$N$_2$

Molecular Weight: 662.66

$\lambda = 713$ nm  $\varepsilon = 39800$
ESS-23

Chemical Formula: C_{33}H_{53}N_{2}O_{2}
Molecular Weight: 614.52
Calculated Elemental Analysis: C, 64.50; H, 5.08; I, 20.65; N, 4.56; O, 5.21
Found Elemental Analysis: C, 64.38; H, 4.61; I, 20.65; N, 4.39; O, 5.21

ESS-16

Chemical Formula: C_{35}H_{44}N_{2}O
Molecular Weight: 680.66
Calculated Elemental Analysis: C, 68.82; H, 6.07; I, 18.64; N, 4.12; O, 2.35
Found Elemental Analysis: C, 68.76; H, 6.06; I, 18.64; N, 4.04; O, 2.35

ESS-13

Chemical Formula: C_{35}H_{43}N_{2}O_{6}
Molecular Weight: 714.63
Calculated Elemental Analysis: C, 58.82; H, 6.06; I, 17.76; N, 3.92; O, 13.43
Found Elemental Analysis: C, 58.68; H, 5.32; I, 17.76; N, 3.69; O, 13.43
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**Analysis**

- **Elements Present:** C, H, N
- **Analyze for:**
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  - Explosive: No
  - M.P.: 185
  - B.P.: V.
  - To be dried: Yes
  - Temp.: V.
  - Time: V.
- **FAX Service:** Yes
- **FAX Phone #:** 404-413-5505
- **Rush Service:** No
- **Phone Service:** No
- **Phone No.:** (SEE CURRENT PRICE LIST)

**Received:** APR 03 2014
**Date Completed:** APR 04 2014
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Elements Present: C, H, N

Hygroscopic □ Explosive □
M.P. 176 - 178 B.P.

To be dried: Yes □ No □
Temp. Vac. Time

FAX Service: 404-413-5505

Rush Service □ (SEE CURRENT PHONE SERVICE PRICE LIST)

Received: APR 03 2014
Date Completed: APR 04 2014

No charge for duplicates

Name: Maged Henary
Date: 4/2/14
# ATLANTIC MICROLAB, INC.

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**Analysis Results**

- Elements Present: C, H, N
- Analyze for:
  - Hygroscopic
  - Explosive
  - M.P. 187
  - B.P.
  - To be dried: Yes
  - Temp. Vac.
  - Time

**Contact Information**

- FAX Service
- FAX Phone #: 404-413-5505
- Rush Service: (SEE CURRENT PRICE LIST)
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- Phone No.

---

**Submitted by**

- Name: Maged Hennay
- Address: 161 Jesse Hill Jr. Dr.
- Contact: G.S.U.
- Email: mhenary@gsu.edu
- Company/School: G.S.U.
- City, State, Zip: Atlanta, GA 30303
- Date: 4/2/14

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**Received**

- APR 03 2014

**Date Completed**

- APR 04 2014

**Remarks:**

- NO CHARGE FOR DUPLICATES
Chemical Shift (ppm):
- 147.45
- 141.95
- 139.42
- 133.01
- 129.12
- 126.44
- 125.27
- 124.70
- 119.40
- 117.78
- 110.98
- 108.28
- 41.96
- 40.60
- 40.39
- 40.19
- 39.98
- 39.76
- 39.56
- 39.35

Chemical Formula: $\text{C}_{25}\text{H}_{23}\text{I}_2\text{N}_2$
Molecular Weight: 478.38
Chemical Formula: C_{29}H_{23}ClN_{2}
Molecular Weight: 512.82
Chemical Formula: C_{27}H_{32}N_{2}
Molecular Weight: 506.43
Chemical Formula: C_{27}H_{27}I\text{N}_2
Molecular Weight: 506.43
Chemical Formula: C_{27}H_{28}ClN_{2}
Molecular Weight: 540.87
Chemical Formula: C_{27}H_{30}ClN_2
Molecular Weight: 540.87
ESS-2-5.001.001.1r.esp

Chemical Formula: C_{31}H_{54}Br_N_2
Molecular Weight: 641.44
Chemical Shift (ppm)

ESS-2-5.002.001.1r.esp

Chemical Formula: C₃H₆BrN₂
Molecular Weight: 641.44
Chemical Formula: C$_3$H$_{34}$ClN$_2$
Molecular Weight: 596.98
Chemical Shift (ppm)

n-Bu

Chemical Formula: C_{33}H_{34}ClN_{2}
Molecular Weight: 596.98
Chemical Formula: C₃H₆ClN₂
Molecular Weight: 596.98
6.3 Appendix C

Chapter 3: $^1$H NMR, $^{13}$C NMR, and HRMS Spectra:

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer.
4-methyl-3-N-methylaminophenol

Chemical Formula: C₈H₁₁NO
Molecular Weight: 137.18
Chemical Formula: C₇H₉NO
Molecular Weight: 123.16
Chemical Formula: C₆H₁₃NO
Molecular Weight: 151.21
Chemical Formula: C₉H₁₃NO
Molecular Weight: 151.21
Chemical Formula: C₉H₁₃NO
Molecular Weight: 151.21
Chemical Formula: $C_{11}H_{17}NO$
Molecular Weight: 179.26
Chemical Formula: $C_8H_{10}N_2O_2$
Molecular Weight: 166.18
Chemical Formula: C₉H₁₂N₂O₂
Molecular Weight: 180.21
3-NO-4-N,N-diethylanisole

Chemical Formula: \(C_{11}H_{16}N_2O_2\)
Molecular Weight: 208.26
Chemical Formula: C_{11}H_{16}N_{2}O_{2}
Molecular Weight: 208.26
4-methyl-3-aminoanisole.001.001.1r.esp

Chemical Formula: C₈H₁₁NO
Molecular Weight: 137.18
Chemical Formula: $C_8H_{11}NO$
Molecular Weight: 137.18
### ESS-61

**Chemical Formula:** \( \text{C}_{18}\text{H}_{18}\text{ClN}_3\text{O} \)

**Molecular Weight:** 303.79

![Chemical Structure](image-url)
Chemical Formula: C_{10}H_{18}ClN_3O
Molecular Weight: 303.79
Chemical Formula: $C_{16}H_{18}ClN_3O_5$
Molecular Weight: 367.79
Chemical Formula: C_{18}H_{18}ClN_{3}O_{5}
Molecular Weight: 367.79
Chemical Formula: C_{16}H_{18}ClN_{3}O_{5}
Molecular Weight: 367.79
ESS-92

Chemical Formula: C_{18}H_{18}ClN_{3}O_{5}
Molecular Weight: 367.79
ESS-56a

Chemical Formula: C₉H₁₃NO
Molecular Weight: 151.21
Chemical Shift (ppm)

7.68 7.66 7.45 7.20
3.91 1.02 1.03 0.98
2.73 2.70 0.95 0.78
6.76 6.75 6.68
7.66
3.69 3.67 3.64
2.37 2.30 1.95 1.93
1.94 1.34 1.31 1.30
1.28 1.26

Chemical Formula: C_{19}H_{24}ClN_{3}O
Molecular Weight: 345.87
Chemical Shift (ppm)

159.09
156.59
150.54
150.16
136.26
134.98
134.23
133.67
131.61
118.69
117.93
97.07
95.09

ESS-102

Chemical Formula: C_{19}H_{24}ClN_2O
Molecular Weight: 345.87
50b

Chemical Formula: C_{20}H_{26}ClN_{3}O_{5}
Molecular Weight: 423.89

Chemical Shift (ppm)
2.49
3.68
1.84
1.72
6.00
4.07
1.96
1.98

M09(br. s.)
M08(s)
M03(t)
M02(br. s.)
M01(t)
M04(t)
M07(m)

7.64
6.93
6.91
3.70
3.68
3.67
3.66
3.64
2.40
1.81
1.79
1.49
1.41
1.39
1.37
1.00
0.98
0.96

Chemical Shift (ppm)
Chemical Shift (ppm)

205.64
205.45
205.25
148.89
132.40
129.12
93.72
93.59
65.22
43.99
39.06
29.54
29.40
29.34
29.15
28.96
28.77
28.58
28.38
16.58
13.19

Chemical Formula: C_{20}H_{28}ClN_{3}O_{5}
Molecular Weight: 423.89
Chemical Formula: C₁₇H₂₀ClN₃O₅
Molecular Weight: 381.81
Chemical Shift (ppm)

M04 (br. s.)
M05 (br. s.)
M02 (br. s.)
M06 (br. s.)
M08 (m)
M03 (d)
M07 (q)

Chemical Formula: C\textsubscript{17}H\textsubscript{20}ClN\textsubscript{3}O\textsubscript{5}
Molecular Weight: 381.81