Modifications To The Benzophenoxazine Architecture: Synthesis And Characterization

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Recommended Citation
doi: https://doi.org/10.57709/8535112
ABSTRACT

Nile red and Nile blue are intensely colored fluorescent dyes from the benzophenoxazine family. Modification of the donor amine with varying alkyl substituents was achieved with the principle aim being to investigate differences, both optical and physical, between dye scaffolds outfitted with secondary amines versus tertiary amines. These changes to the dyes’ architectural framework gave holistic effects to the bulk molecule by modulating hydrophobicity, optical properties, and protein binding constants to human serum albumin.

INDEX WORDS: Benzophenoxazine, Dye, Nile red, Nile blue, Synthesis
MODIFICATIONS TO THE BENZOPHENOXAZINE ARCHITECTURE: SYNTHESIS AND CHARACTERIZATION

by

VINCENT JOHN MARTINEZ

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Masters in Science in the College of Arts and Sciences Georgia State University 2016
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CHARACTERIZATION

by

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May 2016
DEDICATION

I would like to dedicate this manuscript to my mother, Gara Martinez. I would not be where I am without her.
ACKNOWLEDGEMENTS

There have been so many people that have enabled me to pursue graduate training and that have helped me to resolve problems and focus my ideas. I would like to acknowledge my P.I., Dr. Maged Henary, for believing in my talent and for giving my research a direction. I would like to extend my personal thanks to our group’s post-doctoral fellows both past and present, specifically Dr. Yunshan Wu and Dr. Konstantin Zyabrev. They were always very courteous and willing to extend a helping hand. Our team of talented graduate researchers has been an asset to my work. I would like to thank Eduardo Soriano, Carl Kananda, Matthew Laramie, Andrew Levitz, Eric Owens and Cory Holder for helping me to troubleshoot synthetic procedures or to debate mechanistic pathways. I would like to also thank our collaborators Eman Alsolmy and Dr. Gabor Patonay for doing excellent work and being great partners. The staff and faculty at Georgia State have been incredibly helpful. They have kindly taught and mentored me over the years. Lastly, I want to thank Amanda Mauldin for her patience. She has been instrumental in keeping me focused and her loving support has undoubtedly been essential to my success.
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1 INTRODUCTION

Color brings beauty to life. Everywhere we look, our eyes have evolved to see color. Whether it is the gorgeous shades of purple on a bougainvillea’s flower or the striking red of a cardinal’s plumage, color is ubiquitous in this world. But the utility of color is far deeper than pure aesthetics. Biologically, color is used as a signal. It can be a dire warning or a brash advertisement. The caterpillar of the monarch butterfly is a brilliant yellow, a caution to all birds who would dare to eat its poisonous flesh. The beautiful blue and iridescent feathers of the peacock are a signal to potential female mates that he is a male worthy of their attention. If one could see shorter wavelengths of light they would be privy to a world where flowers were not only beautiful but were tattooed with an array of ultraviolet runways. These are to attract bees much like the neon signs of the Vegas strip attract hordes of tourists and gamblers from across the desert vista.

A scientist might see color in a different light. Color is utility. Relationships have been devised that can use color to accurately measure a concentration of a chemical or to tag a biological molecule. What if a biologist wanted to clearly see an organelle deep in the interior of a cell? What if a surgeon wanted to visualize cancerous tissue for removal? What if an analytical chemist wanted to measure the amount of a toxic metal that was in a river or pond? Color can be generated and measured in all of these instances to offer relatively foolproof solutions to real world problems. The workhorse and primary tool for any chemist concerned with color is the dye.

A dye is simply any molecule that can absorb light. It can be differentiated from a pigment which is typically insoluble in most mediums and is of a much larger particle size. A dye can potentially undergo a number of different quantum processes when affected by light. It will always absorb light but some dyes can fluoresce or even phosphoresce. To the layman, the dif-
ference between these phenomena may seem subtle but that there is a complex array of quantum processes that result in these differing behaviors.

1.1 Fluorescence Spectroscopy

Fluorescence phenomena were first observed and documented by Sir Fredrich William Herschel in 1845. He was observing a solution of quinine and noticed that under “certain incidences of light” the solution gave off a “beautiful celestial blue color.”\(^1\) Since then, fluorescence has been studied extensively and is used in all facets of modern science and technology. There are biochemical applications of steady-state anisotropies, distance measuring using resonance energy transfer,\(^2\) mutiphoton microscopy,\(^3\) fluorescence sensing, DNA sequencing, fluorescence-lifetime imaging microscopy, single molecule detection, fluorescence correlation spectroscopy, and a plethora of other fluorescence dependent analytical techniques.

The best tool available for a qualitative description of fluorescence is the Jablonski diagram. The diagram (Figure 1.) clearly illustrates electron mobility between ground and excited energy states. It is most convenient for conveying all of the quantum processes responsible for observed optics. These include absorbance, fluorescence, and phosphorescence which are all dictated by excitation, relaxation, emission, internal conversion, phosphorescence, and intersystem crossing.
1.1.1 Fluorescence vs. Phosphorescence

Valence electrons are the most easily excitable electrons. In the ground energy state, these electrons are located in the highest occupied molecular orbital or HOMO. An absorption event occurs when an incident photon equal in energy to the energy gap between the lowest unoccupied molecular orbital (LUMO) and HOMO strikes the electron. The energy is nearly instantaneously absorbed ($10^{-15}$ s) and the electron is excited across the energy gap from HOMO to LUMO. It should be noted that within individual electronic energy states, there exists a vast multitude of vibrational energy states and within these an even vaster array of rotational energy states. An excited electron will typically be excited to a vibrational state within an excited electronic state. This is called a vibronic transition being a combination of vibrational and electronic.

Figure 1.1— A Jablonski diagram showing the quantum processes related to the absorption and emission of photons
The electron will very quickly relax to the ground vibrational state within the excited electronic state and in so doing will give off energy as heat. This is known as internal conversion. For fluorescence to occur there cannot be any overlap of vibrational states from $S_0$ to $S_1$. If there is overlap then the electron will continue to relax by generating heat. However, if there is no overlap, the only way for the electron to fully relax is to emit a photon of equal energy to the band gap between electronic energy levels. This phenomenon is known as fluorescence.

Fluorescence occurs rapidly, on the order of $10^{-8}$ s. This is still very slow compared to absorption processes. Fluorescence occurs from the relaxation of an electron that is in the singlet state. There are two states that the excited electron can occupy, the singlet state and the triplet state. A good way to understand triplet and singlet is to envision a helium atom. There are only two electrons and if one of them is excited then there are four possible spatial configurations that the electrons may find themselves. In one of the combinations, the electrons have opposite spin vectors making their net spin zero. This is called the singlet state because there is a single orientation with a net spin vector of zero. The other state arises from the other three orientations hence the moniker triplet. If the spins are both aligned up then $S = 1$. If the spins are both aligned down then $S = 1$. If the spins are aligned in opposite directions but are parallel then they will have a net vector perpendicular to their axes of rotation. $S = 1$ in this instance as well.

In most cases it is highly unlikely for an excited electron to enter the triplet state. If such an event occurs, called intersystem crossing, relaxation becomes a forbidden transition. This is because the excited electron and the ground electron from the same ground state orbital will have the same spin vector. The Pauli Exclusion Principle states that no two electrons in the same atom can have the same four quantum numbers, these being $n$, $l$, $m_l$, and $m_s$. If relaxation were to occur from this triplet state then it would be in blatant violation of the Pauli Exclusion Principle.
After a time in the excited triplet state that is orders of magnitude longer than that of the excited singlet state (milliseconds to minutes) the electron will eventually be able to relax. Once again, if there is no overlap between electronic states by vibrational modes then relaxation will occur, not with the release of heat, but with the emission of a photon. An emission event of this sort is called phosphorescence and for the sake of brevity, it will not be discussed any further in this review.

1.1.2 Fluorescence Quenching

Quenching is a mode of action that results in a decrease in fluorescence intensity. There are many different types of quenching and each can give useful information about the fluorophore and how it interacts with its molecular environment. There is collisional (also called dynamic) quenching that occurs when electrons in the excited state lose energy from molecular collisions causing a loss of fluorescence. A well know collisional quencher is molecular oxygen which quenches almost all know fluorophores. Reactions in the excited state, molecular rearrangements, different modes of energy transfer, and formation of a ground state complex can all result in quenching. Heavy atoms such as bromine and iodine can also act as quenchers when they are bound onto a fluorophore’s pi system. These heavy atoms promote intersystem crossing of the excited electron to the triplet state. Due to relaxation times that are orders of magnitude longer in the triplet than in the singlet state, local quenching processes are far more pronounced. So even though the heavy halogens are promoting phosphorescence, they are indirectly causing quenching to occur.

The Stern-Volmer equation directly illustrates a decrease in fluorescence intensity and lifetime due to collisional quenching.

\[
\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q]
\]
Quenching can depopulate the excited state and is a rate process. This is what causes the decreased lifetime.

Static quenching is different from collisional quenching in that as soon as a photon is absorbed by the fluorophore it immediately relaxes without the emission of a photon. This is caused by the formation of a ground state complex with the fluorophore and the quencher. A convenient way to differentiate between dynamic and static quenching is to observe the absorbance spectrum of the molecule. If the quenching mode is dynamic then the absorbance spectrum will remain unperturbed since it is only the excited state electron that is perturbed. However, if the quenching mode is static then the formation of the ground state complex will affect the appearance of the absorbance spectrum.

### 1.1.3 Resonance Energy Transfer

One of the most novel uses of fluorescence is to measure distance. This can be done by a method called resonance energy transfer (RET.) Other accepted names are fluorescence resonance energy transfer or Förster resonance energy transfer (both called FRET.) FRET measures the distance between two dye molecules, termed the donor and the acceptor, by reporting on the amount of non-radiative energy transfer between them. This is done directly by measuring the total intensity and taking a ratio of the acceptor intensity to the total intensity. The formula below gives the efficiency of energy transfer.

\[
E = (1 + \left( \frac{R}{R_0} \right)^6)^{-1}
\]

**Equation 1.2** Efficiency of energy transfer in FRET.
R is the inter-dye distance. $R_0$ is the Förster radius. RET measurements allow real time tracking of a single molecule’s conformational dynamics. RET is now even being used to probe the dynamics of individual DNA and RNA molecules.

1.1.4 Fluorescence Anisotropy

Isotropy is defined as having uniformity in all directions. Anisotropy, on the other hand, is the property of being directionally dependent. When a sample is excited with plane polarized light, the emitted photons also tend to be polarized in most cases. Anisotropy is the term used to describe the extent of polarization of the emitted photons from the sample. There is a reason for this observed polarization. Analyte molecules that have their absorption-transition moments oriented parallel the electric vector of the incident light are more likely to be excited than those that are orthogonally oriented.

Steady state anisotropic measurements can be used to monitor protein binding. The anisotropy of the naked protein can be measured. Then it can be mixed with substrate and the anisotropy can be measured again. The difference in anisotropy is a strong indicator of binding and can be used to calculate the stoichiometry.

1.1.5 Fluorescence Applications

The field of fluorescence microscopy has experienced a renaissance over the last decade or two. One of the most pronounced breakthroughs was the discovery of green fluorescent proteins (GFP) which were isolated from the jellyfish *Aequorea victoria*. The cDNA from these proteins can be concatenated with the DNA of target proteins. The new proteins will retain fluorescent properties while still maintaining the primary functions and cellular localizations of the native protein. This has remarkable implications for using microscopy techniques to actually visualize the inner workings of living tissue in real time.
A classic technique that is arguably the cornerstone fluorescence microscopy technique is confocal scanning microscopy. Thin samples are easily excited with light and basic fluorescent microscopy techniques can be employed to great effect. However, whenever a thick sample is irradiated with light all areas are excited indiscriminately. A great deal of excited photons that are detected come from these out of focus fluorophores. Confocal microscopy seeks to rectify this issue by only allowing light to be detected that originates from the focal point.\(^9\)

Out of focus light is eliminated by using optical sectioning. A large number of images are taken of each cross section of the surface of the object being observed. This is the scanning action described in the method name. A computer is then used to take all of the images and to reconstruct a three dimensional image of the object.\(^9\)

Another technique that at one time was merely thought of as thought experiment by the famous German-American physicist Maria Goeppert-Mayer is two-photon microscopy.\(^7,10\) A major pitfall when attempting to image a biological system is that biological tissues scatter light.\(^11\) Two-photon microscopy allows biologists to actually image this tissue by offering unprecedented optical penetration. Most traditional forms of fluorescence-based microscopy such as confocal microscopy use single-photon excitation. In order for two-photon excitation to occur, the lasers being employed must be incredibly fast. The limit set to where two photons are said to be arriving simultaneously is under five femtoseconds\(^11\) and todays ultra-fast lasers can fire in femtosecond pulses. The energies of the two photons are summed and are simultaneously absorbed by the chromophore. Excitation and emission are not changed by this technique. This greatly enhances the efficiency of fluorescence microscopy where light intensities are sometime too low to cause a sizeable amount of excitation events. A key aspect in determining the efficiency of two-photon excitation is the multiphoton absorption cross-section. The light typically
used is in the near infrared which is deeply penetrating of tissue and is scattered far less than visible light.

Fiber-optic fluorescence imaging has recently received a boost in overall applicability from the development of a “photonic crystal fiber that can facilitate ultra-short pulse delivery for fiber-optic two-photon fluorescence imaging”\(^\text{12}\). Fiber optics allows the user access to cell types that are not normally available to image, especially in vivo. These include tissue cavities and the insides of solid organs.\(^\text{12}\) Due to the continuing shrinking of solid fiber optic parts, both optical and mechanical, and the use of deeply penetrating two-photon excitation techniques for microscopy, the use of fiber optics is now allowing unprecedented access into living organisms to view life as it happens.

1.2 Introduction to Nile Red and Nile blue Dyes

One of the earliest drivers for the formation of the modern chemical industry was the demand for synthetic dyes for the artificial coloring of fabrics. Small-molecule dyes have since found use in a wide range of industrial applications and cutting edge technologies. There are current needs being fulfilled in solar cells, \textit{in vivo} imaging, photodynamic therapy, histological staining, metal sensing, organic light emitting diodes (OLED), and in laser pumping mediums.\(^\text{13-30}\) Many of the popular chromophores such as carbocyanines, BODIPY, coumarin, squaraine, and rhodamine-based dyes are used across academic research, medicine and industry.\(^\text{31-35}\) All dye architectures have their benefits and drawbacks related to photo/chemical stability, optical properties, solubility and synthetic access to analogues.

Benzophenoxazine dyes are often recognized in the literature for their intense colors and their lipophilic nature. Nile red and Nile blue are two fluorescent molecules belonging to this
benzophenoxazine family and historically, they have been used to dye clothing and stain lipids *in vitro*. Since their synthesis was first reported by Möhlau and Uhlmann in 1896, there have been ample papers published on the dyes’ properties and uses.\(^{36}\) Benzo[\(a\)]phenoxazines are analogous to benz[\(a\)]anthracene, also known as tetracehne, but with two heteroatoms, nitrogen and oxygen. This compact aromatic structure is noted for its strong fluorescence and stability.

![Figure 1.2 Structures of Nile red and Nile blue](image)

Nile red has no formal charges and is intensely fluorescent with a high quantum yield in apolar media. However, in water, the dye is poorly soluble and does not show any fluorescence. Its chromophore is highly susceptible to changes in solvent polarity and dielectric constant giving this dye a wide solvatochromic range. Nile blue is a cationic dye and is thus more readily soluble in water than Nile red. It is highly sensitive to pH changes making it a useful pH probe. It has red shifted absorbance spectra compared to Nile red making it a viable candidate for biological imaging.

### 1.3 Nomenclature of oxazines

The name benzophenoxazine is derived from oxazine which corresponds to any heterocycle that contains both oxygen and nitrogen in a six-membered ring with two double bonds.\(^{37}\) There are three possible isomers based on the position of the heteroatoms. They are 1,2-oxazine, 1,3-oxazine and 1,4-oxazine. Elongation of 1,4-oxazine by substituting two fused benzene rings along its only two C—C faces yields a phenoxazine with a similar linear shape to that of anthra-
cene. Further elongation of the conjugated system with another benzene ring fused along the $a$-$c$ faces will give a benzophenoxazine. Nile red dyes are frequently referred to as benzo[a]phenoxazinones while Nile blue are called benzo[a]phenoxaziniums with a counter ion appended on as a suffix. These counter ions are typically halides but more exotic ions such as perchlorates are reported.

Figure 1.3 Nomenclature of oxazines

1.4 Photophysical Properties

Absorbance maxima are determined by the frequency of light required to excite an electron across a HOMO/LUMO band. The energy required for a $\pi \rightarrow \pi^*$ transition is in the long UV through visible range wavelengths and can even extend into the near-infrared part of the spectrum. Benzo[a]phenoxazinones (Nile red) have absorption maxima at 550 nm in methanol while
benzo[a]phenoxaziniums (Nile blue) absorb at 630 nm. Why is there such a large difference between the two similar systems? The 80 nm difference can be accounted for in the strength of the two dyes’ corresponding donor-acceptor systems.

Nile red is a neutral molecule with an amine donor and an oxo (carbonyl) acceptor. Intramolecular charge transfer must occur from a twisted excited state so as to attain overlap of the donor and acceptor molecular orbitals. Nile blue, which is cationic, has a much stronger acceptor in the form of the charged iminium group. This induces a strong net dipole across the molecule driving resonance. Because of this, the sp\(^2\) character of the donor amine is more pronounced than in the neutral system and intramolecular charge transfer is more fluid. The physical implications of this are that the electronic band gaps for Nile blue-based dyes are smaller than those for Nile red-based dyes. Increased delocalization of electrons through intramolecular charge transfer processes will give a sizeable bathochromic shift.

In water, the photophysical behavior of Nile blue is most impacted by the acid form of the dye which can form H aggregates and do not fluoresce. It was found that small substitutional changes to the dye core had a measureable effect on acid/base equilibria with respect to the ability of the solvent’s molecules to act as hydrogen bond acceptors. Alkyl groups substituted on the acceptor iminium cation can lend electron density to the N—H bond via inductive effects. This increases the strength of subsequently formed hydrogen bonds, thus decreasing the proton’s acidity. The deprotonated dye species experience hypsochromic shifts of approximately 100 nm and are an order of magnitude less fluorescent than the acid forms.\(^{38}\)

Emission spectra provide a window into the fine electronic band structure of a fluorophore and provide a glut of information regarding intermolecular interactions. Both benzo[a]phenoxazinones and benzo[a]phenoxaziniums are notable for their high fluorescence quan-
tum yields. The compact chromophore is held in a rigid conformation due to the multiple fused ring systems. Vibrational modes are highly restricted in these systems so the chances of thermal deactivation of the excited state are significantly diminished.

1.4.1 Solvatochromism

Steady-state spectral data has shown that a large change in dipole moment occurs upon excitation of Nile red.\textsuperscript{39} This trait is caused by a process known as twisted intramolecular charge transfer (TICT). Nile red has an amino group at the 9-position which is substituted with two ethyl groups. This amine can rotate around the axis of the sigma bond appending it to the chromophore.

![Figure 1.4](image) A) Nile red in electronic ground state (planar); B) Nile red in in twisted excited state.
It has been demonstrated by Adamo et al. that this bond rotation greatly increases the dipole moment across the entire molecule and that intramolecular charge transfer (ICT) is far more pronounced in this twisted state. This is because the amine donor and oxo acceptor groups have overlap of their molecular orbitals. This hybridizes the donor/acceptor electronic states thus promoting ICT. Because the twisted excited state is so polar, it is best stabilized in polar solvents. In nonpolar media, TICT cannot occur because the Franck-Condon excited state cannot be stabilized due to its large dipole. There is nothing in the molecule’s solvation sphere to stabilize such an excited state. Because of this, the molecule will maintain planarity even once excited. The trend is that increasing solvent polarity causes the absorption maximum to shift bathochromically while decreasing solvent polarity causes the absorption maximum to shift hypsochromically. This is directly correlated to having a higher energy Franck-Condon excited state versus a lower energy one. Time-dependent density functional theory (TD-DFT) was used to computationally predict solvent effects on optical spectra for Nile red and Nile blue with results that closely mirrored the experimental data sets.

Nile red’s TICT processes have been assessed in reverse micelles, Langmuir-Blodgett films, and faujasite zeolite in order to understand how the dye’s photophysical properties were affected once constrained onto a surface that would restrict its geometry. Quantum yield and other optical properties have also been assessed in solution with various organic bases. The dye’s powerful solvatochromism has been used to probe the partitioning of surfactants into aqueous and organic phases. Studies have been done on solvent effects on Nile red using many approaches. Solvatochromism studies of Nile red and Nile blue in isotropic mediums versus anisotropic mediums were also conducted.
1.4.2 Thermochromism

Thermochromism is a molecule’s ability to undergo changes in its optical properties by varying the temperature. Perhaps the most notable example of this phenomenon is the commercial use of thermochromic inks by the Coors Brewing Company to print labels on their beer.\textsuperscript{52}

Kawski \textit{et al.} determined the excited state dipole moment of Nile red in several solvents. They used thermochromic shifts in optical spectra as a way of measuring these parameters. They cite a discrepancy in the literature saying that other researchers publishing in this field neglected to take into account the Onsager reaction field for a polarizable dipole. They claim that their method of measurement gives dipole values for the single isolated molecule irrespective of solvent properties and interactions.\textsuperscript{53}

1.4.3 FRET

Förster resonance energy transfer (FRET) is a photophysical phenomenon whereby an excited state chromophore can excite a neighboring chromophore by through-space energy transfer. This is facilitated by overlap of the FRET donor emission band with the FRET acceptor absorption band in conjunction with the parallel spatial orientation of the donor’s HOMO wave function with that of the acceptor’s LUMO wave function. Within a short enough distance, known as the Förster distance, resonant energy transfer is accomplished vis-à-vis the FRET donor’s excited-state polarization. Electron spin coupling at close range gives rise to the acceptor being able to “feel” the donor. As the donor’s excited singlet state electron attempts to relax, the switch in polarization is felt by the spin coupled electron which then gains the kinetic energy needed to become promoted to an excited state. Chemists can utilize this process to attain a very small molecular ruler allowing measurements to be made on the Angstrom scale.\textsuperscript{54}
Broumische et al. designed a bichromic FRET system with a large two-photon absorbing (TPA) cross-section. Irradiation of the of the donor segment with high intensity IR radiation will give two-photon absorption followed by resonance energy transfer to the Nile red acceptor.\textsuperscript{55}

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{image.png}
\caption{Nile red conjugated to moiety with large TPA cross section. Excitation with 815 nm, 8 ns laser pulses gives near complete quenching of donor fluorescence with notable acceptor fluorescence.}
\end{figure}

They followed up that paper with another publication showing multiphoton absorption by several dendritic structures followed by FRET to a Nile red acceptor.\textsuperscript{56} This carries significant advantages by allowing an attractive UV-vis absorbing chromophore with a small TPA cross section to be excited by IR radiation which would otherwise not be feasible.

1.4.4 Intersystem Crossing

Heavy atoms have a long reported history of causing photophysical and photochemical intramolecular effects in organic molecules.\textsuperscript{57, 58} Generation of singlet oxygen is promulgated by through-space energy transfer from neighboring electrons in the excited triplet state. Since the
heavy atom effect is efficient at generating large populations of excited triplet electrons, it is also quite effective at generating singlet oxygen.

Singlet oxygen is highly reactive and is utilized as a destructive agent in photodynamic therapy (PDT) whereby cancerous tissue is bombarded with high concentrations of singlet oxygen generated \textit{in situ} by excitation of a PDT agent with light. Benzo[\textit{a}]phenoxazines are poor candidates for PDT but derivatization with heavy halogens and chalcogens can yield more desirable candidates. Substitution of the oxazine’s oxygen with a sulfur or a selenium shows a dramatic increase in singlet oxygen yields as shown in Table 1.1.

![Figure 1.6 Benzo[\textit{a}]phenoxazine analogues with different chalcogens substituted at the 7-position.](image)

<table>
<thead>
<tr>
<th>Dye</th>
<th>(\lambda_{\text{abs}}) (nm)\textsuperscript{[a]}</th>
<th>(\lambda_{\text{em}}) (nm)\textsuperscript{[b]}</th>
<th>(\Phi)\textsuperscript{[c]}</th>
<th>(^1\text{O}_2)\textsuperscript{[d]}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>632</td>
<td>660</td>
<td>0.27</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>654</td>
<td>693</td>
<td>0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>661</td>
<td>703</td>
<td>0.03</td>
<td>0.78</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} Absorption maximum in ethanol containing 0.1% acetic acid. \textsuperscript{[b]} Fluorescence maximum in ethanol. \textsuperscript{[c]} Absolute fluorescence quantum yield. \textsuperscript{[d]} Absolute quantum yield for single oxygen formation.

\textbf{Table 1.1} Photophysical properties of Nile blue analogues with differing chalcogen substitutions.
1.5 Applications of Nile Red and Nile Blue

Nile red and Nile blue have found many uses in industry and science since their original syntheses in the late 19th century. Their intense fluorescence and good photostability have made them attractive options as sensors, photosensitizers, and biological labels.

1.5.1 Materials Science Applications

1.5.1.1 Loading onto Inert Supports

Dyes incorporated into materials or onto surfaces can act as sensors for a variety of applications. Nile blue was infused with poly(n-butyl acrylate) microspheres to be used as a reflectometric ion sensor for potassium. Nile red has been embedded in polystyrene films on a glass surface and adsorbed onto nanoclays to assess the hydrophobic loading abilities of these clays. It has been tested for dye-sensitized solar cells. It has also been incorporated into graphene sheets for potential use as a biosensor or non-palladium fuel cell catalyst. Nile blue, loaded into a silica sol gel matrix, was investigated for use in a solid state dye laser.

Huang et al. wanted to study the electronic bands and transitions of Nile blue. They constructed Langmuir-Blodgett films with the dye embedded and then deposited these onto clay sheets. By changing the concentration of dye and the surface pressure, the group was able to form two types of adsorbed states. One state (type I) consisted of dyes stacked face to face and had an absorption maximum at 550 nm. This is very similar to the dye in solution with methanol. The other state (type II) consisted of the dyes stacked side-to-side with their long axes oriented perpendicular to the air/water surface. These had an absorption maximum of 590 nm.
1.5.1.2 Nanoparticles

Nanoparticles have been a hot topic for many years. Gold nanoparticles are known for having massive Stokes shifts. Nile red can adsorb to their surface which will then weakly fluoresce due to FRET. Thiols in solution can compete with Nile red for adsorption sites giving a change in fluorescence and this is selective against amines, acids, alcohols, bovine serum albumin (BSA), and hemoglobin making it a useful detection method for thiol containing biomolecules.66-70

Gold nanoparticles doped into a polymer matrix containing Nile blue can have a direct effect on the dye’s luminescence. Through surface plasmon coupling, luminescence can be enhanced by changing the distance of the dye molecules from the gold nanoparticles and by altering the charge on the gold nanoparticles. The excitation wavelength of the pumping laser source plays a strong role in this enhancement.71 Nile red has recently been used in a polymer nanoparticle with a PEGylated exterior to selectively sense Fe$^{3+}$ ions.72

1.5.1.3 Miscellaneous

Research during the early days of World War II was undertaken to search for acid/base indicators for use in non-aqueous media. The applications of such a dye could be for the detection of acid in essential war materials such as airplane fuels and lubricants. By 1966, this research had led to the investigation of Nile blue A as a good candidate for such detection. The anhydro form of the dye Nile blue is relatively basic since protonation results in a stable, cationic charge transfer platform. Nile blue was found to be well suited for titrations in apolar media.73

The EPA uses a variety of field tests for rapid detection and evaluation for a variety of toxins and contaminants in water and soil. Duan et al. were seeking to develop to a probe for the rapid detection of hypochlorite in water. Other probes and methods already exist for this applica-
tion but they wanted a method that used a cheap, commercially available fluorescent dye. They selected Nile blue A which had its fluorescence quenched in the presence of hypochlorite. The detection limit was very low (0.04 μmol/L) and appeared specific to hypochlorite ions making detection facile for trace quantities of hypochlorite in water samples.\textsuperscript{74}

1.5.2 Biological Applications

1.5.2.1 Biological Imaging

Nile red has been used historically as a histochemical stain to image lipid droplets in tissue.\textsuperscript{75,76} Dioctyldecyldimethyl- ammonium bromide (DODAB) is a cationic lipid that composes some liposomes. By measuring the steady-state and time resolved anisotropy of Nile red, one can monitor the distribution of cholesterol in these DODAB vesicles. Fluorescence also reported useful information about the viscosity and polarity of local microenvironments within the vesicle.\textsuperscript{77}

Nile blue also has an affinity for lipophilic molecules. Nile blue localizes in lysosomes and can subsequently be taken up by tumor cells.\textsuperscript{78} Nile blue was investigated for use as a non-porphyrin photosensitizer.\textsuperscript{79} Its effects were studied in human bladder carcinoma cells for use as an agent for photodynamic therapy (PDT).\textsuperscript{80}

1.5.2.2 Biological Assays

Another common use for fluorescent dyes is in assays. Mild hypothermia (mHT) induced in tumor cells has been shown to increase the bioavailability of low molecular weight drugs due to a multitude of overlapping factors such as increased vascular permeability and tumor perfusion. The human epidermal growth factor receptor 2 (HER-2) overexpressed in breast cancer can be targeted effectively by a clinically approved antibody, Herceptin\textsuperscript{®}. Escoffre et al. used a Nile
red assay to measure the formation of Herceptin® aggregates in order to assess the stability of the drug under mHT conditions.⁸¹

Nile blue was also used to study the efficacy of new drug release technology.⁸² Mishra et al. investigated the binding of Nile blue to bile salts. Bile salts act of biosurfactants and are able to solvate a variety of hydrophobic biological chemical structures such as cholesterol and lipids. They have garnered interest as a potential drug delivery vehicle due to their low toxicity and biocompatibility.⁸³

Kuramitz et al. developed a binding assay for protein-ligand interactions. They used spectroelectrochemical sensing which combines both optical spectroscopy and electrochemical techniques. With these two detection modes successfully integrated into a probe, the accuracy of measurements is increased due to the corroboration between the two data sets. They chose Nile blue as the optical label for the spectroscopic portion of their sensor.⁸⁴

1.5.2.3 Biological Sensors

Due to the pH sensitivity of the Nile blue chromophore, it has been the subject of testing for use as a pH probe. Ma et al. used fluorescein isothiocyanate (FITC) and Nile blue chloride to construct a ratiometric pH sensitive probe. The FITC responds to small changes in pH. Increasing pH will cause the FITC emission signal at 515 nm to undergo a hyperchromic shift. Nile blue fluoresces at 670 nm and the signal stayed relatively constant through minor pH manipulations. This allowed the two emission spectra to be compared so as to determine the pH ratiometrically. FITC was chemically conjugated to the surface of carbon quantum dots. In order to keep Nile blue chloride from diffusing off of the surface, the quantum dots were coated in a sodium alginate hydrogel that would immobilize the dyes from “leaking”. This sensor was tested for tracking the pH of the extracellular fluid between cancer cells in real time.⁸⁵
Nile blue-based platforms for urea biosensors have been constructed.\textsuperscript{86, 87} One such method involves a sensor design that was based on immobilizing a Nile blue chromoionophore and a urease enzyme in stacked sol-gel films. This method does not require chemical conjugation for analytical detection applications. The other method uses a kappa-carrageenan membrane that was doped with a Nile blue chromoionophore as an optode. Urease was physically attached to this structure for urea sensing.

\textbf{Figure 1.7} Nile blue being reduced by alkaline phosphatase enzyme to yield a non-fluorescent product.

Surface-enhanced Raman scattering (SERS) can be used to detect alkaline phosphatase. Nile blue A is Raman active and they used it to replace nitro blue tetrazolium which is more commonly used. The oxidized form is Raman active and it can be reduced by the ALP substrate product effectively turning off signal.\textsuperscript{88}
1.5.2.4 DNA Applications

Nile blue binds to DNA. Mitra et al. looked at the binding of Nile blue with three different synthetic DNA oligonucleotides: (CGCAAATTTGCG)$_2$, (GCGGCGGCGGCG)$_2$, and (ATATATATATAT)$_2$. Using picosecond resolved fluorescence quenching and polarization analyzed anisotropy they were able to identify two modes of binding between the dye and DNA. Both modes were concentration dependent. Low DNA concentrations saw electrostatics play a large role in dye-DNA binding. At high concentrations of DNA, the preferred mode was intercalation of DNA by the dyes.$^{89}$

Nile blue undergoes static quenching upon binding with DNA. This quenching is linear over a wide concentration range of nucleic acids and serves as the basis for a fluorescence quenching assay for the determination of concentrations of DNA and RNA down to ng•mL$^{-1}$ levels.$^{90}$ There is also a protocol for the determination of DNA concentrations from the three-dimensional spectra of light scattering from Nile blue sulfate on DNA.$^{91}$ Studies of the binding of Nile blue sulfate to calf thymus DNA and fish sperm DNA showed through absorbance spectra that it was the H aggregates that were that were doing the binding; this process was enhanced in the presence of single stranded nucleic acids such as native yeast RNA and thermally denatured DNA.$^{92}$

Since the dye has been shown to bind with DNA, a large amount of work has been done to expand the probe’s utility with nucleotides. Staining/histology has historically been popular with benzo[a]phenoxazines. With respect to this history, a postelectrophoretic method for staining DNA with Nile blue in agarose and polyacrylamide gels was developed, adding yet another layer to the dyes histochemical applications.$^{93}$

Another method for DNA detection involves glutathione (GSH)-capped CdTe quantum dots (QDs). They have intense fluorescence and surface binding (noncovalent) of Nile blue to the
QDs would quench fluorescence due to an electron transfer mechanism between the glutathione and Nile blue. If DNA is present in solution, the Nile blue will dissociate from the QDs and bind to DNA causing fluorescence to be restored. This is an on/off capability.\textsuperscript{94} The interactions between oligonucleotides and Nile blue were investigated for the electrochemical detection of hepatitis C 3a virus.\textsuperscript{95}

Nile blue sulfate was put into a DNA-based solid electrolyte for potential application in electrochromic devices such as smart windows.\textsuperscript{96}

1.6 Synthesis of Nile Blue and Nile Red Analogues

Nile red was originally synthesized by boiling Nile blue in sulfuric acid, which would convert the iminium moiety to an oxo group as shown in Equation 2.\textsuperscript{36} This harsh method is outdated and the most frequently reported synthetic protocols call for the use of a nitrosylated precursor instead.

\textbf{Equation 2.} Original method for synthesis of Nile red.

![Synthesis of Nile Red](image)

Synthetic access to analogues is limited primarily to two or three positions on the chromophore. On Nile blue they are the 2-position and the 5- and 9-amino/iminium groups. On Nile red they are the 2-position and 9-amino group (Figure 1.8.). The 2-position is frequently functionalized with a phenol. This can be conjugated to \textit{n}-alkyl chains that are often capped with esters, acids, amines or halides. Many of these are reactive intermediates that can be used in some form of conjugation. Dye precursors contain a primary amine which can be successfully modified via
facile $N,N$-dialkylation reactions to form a large set of analogues. $N$-alkylation and Michael addition are also reported options for functionalization of the amines.

![Nile red and Nile blue scaffolds](image)

**Figure 1.8** Common positions for synthetic modification of Nile red and Nile blue.

Other positions such as the 1, 3, 4, 6, and 8 carbons have been reported to be functionalized but these are typically unique cases without further synthetic development to expand analogue sets. The 10-position is sometimes substituted with a methyl group to aid in the formation of secondary amines at the 9-position by acting as a steric blocker to hinder overalkylation of the amine.

Nile blue is a charged, cationic species and can be prepared in condensation reactions where the anion is dependent on the acid used in its preparation. Chlorides are very common anions but perchlorates have much better solubility. This is because perchlorate is a weakly ligating anion due to extensive delocalization of its anionic charge. This trait coupled with its large size gives the ion very low charge density causing it to have weak associations with its neighboring cations.97
1.6.1 *Dye Synthesis via a Nitroso intermediate*

![Diagram of dye synthesis](image)

**Figure 1.9** General synthesis of Nile red and Nile blue analogues.

The most common method reported for preparing these Nile red and Nile blue dyes starts with alkylating an aminophenol. The primary amine at the 3-position is a good nucleophile and will rapidly attack most alkyl halides. It can also undergo a Michael-like addition with acrylic acid to gain propionic acid substituents. Alkylation this way is seldom clean due to the formation of multiple alkylated products. Column chromatography is almost always required to isolate the desired compounds.

Nitrosylating an *N,N*-dialkylated-3-amino phenol is the next step. This is done by dissolving the substituted aminophenol in a polar solvent, usually a low molecular weight alcohol, with hydrochloric acid added. Sodium nitrite, dissolved in water, is very carefully dripped into the solution. The whole reaction is kept on an ice bath to prevent a runaway exotherm.
Under the acidic conditions, the nitrite anion is dehydrated rapidly to form the nitrosonium cation, a potent electrophilic species. The amine, being the most nucleophilic functionality present in solution, will attack the nitrosonium cation forming a nitroamine. However, the nitroso group will not remain at this locale as it is always directed to the position ortho to the phenol and para to the amine by undergoing Fischer-Hepp rearrangement upon heating. The final product is an HCl salt. These aromatic nitroso groups are very reactive. Heating of a nitrosylated aminophenol under acidic conditions with hydroxynaphthalene or aminonaphthalene will yield Nile red and Nile blue, respectively, via an acid-catalyzed condensation reaction. The whole synthetic process is outlined in Figure 1.9.

Nile blue and Nile red exhibit intense fluorescence so their main utility comes from being able to label, detect and track things. The major problem with these dye systems is that they suffer from a lack of water solubility. This is of paramount concern for any biological applications whether in vitro or in vivo. They also lack good handles for bioconjugation.

There have been several approaches to these problems. Water solubility can be remedied by incorporating sulfonic and carboxylic acids, hydroxyl groups, or even polyethylene glycol (PEG). All of these water soluble analogues have larger fluorescence quantum yields in aqueous media than their insoluble cousins and they are all prepared from nitrosylated aromatics as shown in Figure 1.9.

1.6.2 Dye Synthesis via an Azo Intermediate

Ho et al. synthesized a series of water soluble Nile blue analogues for use in the detection of enzymatic activities. As sulfonate groups were added to the dye, fluorescence intensity increased noticeably in water. This is one of the few instances where a nitroso compound was not used as a dye precursor. The reasoning behind using an azo intermediate lies in the fact that
that sulfonated nitrosoanilines perform poorly in the final dye reaction. Direct sulfonation of the dye is not desirable either as there are multiple aryl positions that are vulnerable to substitution. This greatly complicates the purification process and yields will suffer.

The azo method starts with $p$-nitroaniline which is converted into the diazonium salt 12 in nitrous acid. The diazonium salt is further reacted in situ with either a, b, or c in Figure 1.10 which will then constitute either the western or eastern portions of the final dye. The product is an arylazo compound which can then be condensed with either aminonaphthalene or a 3-aminophenol derivative in the presence of perchloric acid to yield a Nile blue analogue.
Figure 1.10 Synthesis of water soluble Nile blue analogues via arylazo intermediates.

1.6.3 Dye Derivatives with Conjugateable Groups

Organic dyes are typically flat and hydrophobic compounds. They possess few of the traits that one would find in a typical molecule that would be deemed drug like. This generally makes them poor candidates for the selective targeting of biological systems. However, conjugating a dye to bioactive compounds known to penetrate or target specific sites can be invaluable for imparting bioselectivity to a fluorophore. Targets can be lit up with some predictability. Nile red and Nile
blue can both be modified to have functional groups that act as handles. These handles can easily be conjugated to a substrate by one of several well-known methods.

### 1.6.4 Esters and Carboxylic Acids

Carboxylic acids are convenient functional groups for bioorthogonal conjugation so it would be pertinent to incorporate this functionality onto the dye backbone. Substitution with alkyl-capped esters is generally the first step to preparing the acid and this has been accomplished on a vast array of Nile blue and Nile red analogues.\(^{38, 101, 102}\) Many successful attempts have already been made to incorporate an alkyl-capped carboxylic acid at the 5-amino and 9-amino position for Nile blue and the 9-amino position for Nile red.\(^{38, 99, 100, 104}\)

A hydroxyl handle is easily achieved at the 2-position via condensation of the nitrosylated 3-aminophenol with 1-amino-6-hydroxynaphthalene or 1,6-dihydroxynaphthalene for Nile blue or Nile red respectively as shown in Figure 1.9. Substitution at this position is facile with an alkyl linker that has an ester endcap. This can be achieved in two ways. The first method involves alkylating 16 at the 2-hydroxy position with an ester-capped alkyl halide under basic conditions to give 17. Treatment of the ester with pig liver esterase (PLE) at a pH of 7 will deprotect and give the acid. This has a lower overall yield than the second method as is shown in Figure 1.11.

The second method uses a similar alkyl halide but it is capped with a benzyl ester instead of an ethyl ester. The benzyl group is cleaved with the boron trifluoride dimethylsulfide adduct. This gives a 74% yield versus 62% for the PLE deprotection of the ethyl ester.\(^{105, 106}\) If a methyl ester is used, deprotection with potassium trimethylsilanolate (TMSOK) will give very high yields (92%) of the acid.
Figure 1.11 Synthesis of bioconjugateable Nile red derivative with carboxylic acid handle.

\( \text{N-hydroxysuccinimide (NHS) and N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate (morpho CDI) will react with a naked acid to form an NHS ester. Morpho CDI is a carbodiimide salt. The carbodiimides are very effective at forming amide bonds between acids and amines and will condense the naked acid with the NHS. Linkage to a primary amine is facile and as proof of concept, Briggs et al. used allylamine and 3-phenyl-1-aminopropane to form amide links to the dye as is shown in Figure 1.12.}^{105} \) 4-Dimethylaminopyridine, known as DMAP, is a very strong organic base that will catalyze this reaction.
Figure 1.12 Synthesis of amide coupled Nile red analogues to show proof of concept for bioconjugation. All dye—linker bonds are at the 2-position on Nile red. See Compound 19 on Figure 1.12 for complete structure of precursor.

1.6.5 Primary Amines

Just as acids can be used as handles to couple dyes to primary amines, the reverse is also true. Many Nile blue and Nile red derivatives have been prepared with primary amines covalently attached to sp3 carbons.101, 102, 107-109 These are convenient functional groups for coupling and will readily react with a carboxylic acid that has been activated by a carbodiimide.

Figure 1.13 Nile blue and Nile red analogues with primary amine handles attached to the chromophore via alkyl linkers at the 2- and 5-positions.
A convenient method for stapling peptides to each other or onto various structures is through DCC/HOobt coupling. Mild conditions allow an amide linkage to be formed between a carboxylic acid and a primary amine. It has been demonstrated that the technique can be used to append short peptides onto Nile blue. This can be done to a dye precursor before the final condensation reaction to form the dye or it can be achieved directly to the dye itself in good yield.

As shown by Frade et al., DCC coupling can be done to the naphthalene precursor and then condensed to form the dye. Nile blue is also stable enough to undergo DCC coupling with amino acids directly to the dye core. They successfully conjugated the dyes to several amino acids via amide and ester linkages.\textsuperscript{110,111}

![Diagram of synthesis of Nile blue analogues and labeling L-alanine, L-glycine, L-phenylalanine and L-glutamic acid (protected as methyl ester) with them.](image)

**Figure 1.14** Synthesis of Nile blue analogues and labeling L-alanine, L-glycine, L-phenylalanine and L-glutamic acid (protected as methyl ester) with them.
The technique of coupling an amine to an acid has been used to append substrates onto Nile blue for selective targeting. Cyclooxygenase-2 (COX-2) is an enzyme that is overexpressed in tumor cells.\textsuperscript{112-117} Wang \textit{et al.} designed a Nile blue-based NIR probe that has the indomethacin (IMC) moiety conjugated to it via a hexanediamine linker.\textsuperscript{109} This was achieved similarly to the stapled peptides using HOBr. The long linker was used so as to allow the IMC group to fully insert into the COX-2 active site which accumulates in the Golgi of tumor cells. IMC has been shown to bind very tightly and selectively to COX-2. The probe allows for effective NIR imaging of tumors and could have \textit{in vivo} applications.

Preparation of the IMC-linked Nile blue derivative started with the electrophilic aromatic substitution of hexanediamine onto bromonaphthalene as shown in Figure 1.15 to afford compound \textbf{33}. Condensation of this compound with the nitrosylated aminophenol shown will give the dye \textbf{34}. This reaction was discussed in detail in section 4.1. Dye \textbf{34} can be directly coupled to IMC using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCl) and HOBr to yield \textbf{35}.

\begin{center}
\textbf{Figure 1.15} Preparation of a Nile blue-IMC linked derivative with a hexanediamine linker.
\end{center}
Frade et al. reported the synthesis of Nile blue analogues with a series of interesting structures such as pyrene, p-toluenesulfonamide, and an azo moiety. All of these were coupled to a diamine linker extending from the 5-amino position using DCC/HObt. This illustrates further synthetic utility of the method for appending a diverse set of functionalities to Nile blue.

![Figure 1.16 Nile blue analogues with amide linked moieties.](image)

1.6.6 **Azide-alkyne Huisgen Cycloaddition**

Vilaivan et al. used click chemistry to conjugate a Nile red label to pyrrolidinyl peptide nucleic acid (PNA). This was done in order to probe the local environment of PNA-DNA duplexes by utilizing Nile red’s solvatochromic properties.

2-hydroxy Nile red (16) will undergo a simple S_N2 reaction with propargyl bromide in the presence of a mild base such as potassium carbonate in a polar aprotic solvent to give 37 as shown in Figure 1.17. This primary alkyne will undergo a copper-catalyzed “click” reac-
tion with an azide. PNA can have such a group appended on via an alkyl linker and then it can be “clicked” together with the alkyne on 37 to give the Nile red linked PNA (38).

![Figure 1.17 Synthesis of propargylated Nile red and click to acpcPNA.](image)

1.6.7  Halogenated

1.6.7.1  Fluorinated Nile Red Derivatives

Halogens can have many marked effects when incorporated into dye structures. With the exception of fluorine, they make great leaving groups and the heavier ones, namely bromine and iodine, are adept at promoting intersystem crossing of the excited singlet state into the excited triplet state. Fluorine itself is a remarkable atom when substituted for hydrogen on drugable molecules. Its small size makes it a great mimic of hydrogen and its very strong electronegativity can allow it to impart unique effects on a drug without significantly changing its molecular footprint. Park et al. synthesized a series of Nile red analogues with two different polyfluorinated olefin moieties at the 2- and 3- hydroxyl positions. Their series included $N,N$-diethyl as well as $N,N$-dibutyl substituents at the 9-amino position and $n$-butoxy / $t$-butoxy substitutions (not shown) at the 6-position. Only one product is shown for the sake of brevity.\textsuperscript{119}
Gerasimova et al. reported the synthesis of a polyfluorinated Nile red analogue through an $N$-oxide intermediate as shown in Figure 1.19. They started with octafluoronaphthalene and condensed it with 3-$(N,N$-diethylamino)phenol. The product was obtained in a modest yield of 24%. The following step used sodium nitrite and acidic conditions followed by oxidation with O$_2$ gas to generate the $N$-oxide. It was in this step that the yield was abysmally poor with a reported yield of 3%. The final step involved the reduction of the $N$-oxide with zinc under acidic conditions. The overall yield after three steps was a synthetically useless 0.5%.

Ektova et al. sought to improve the yield of Gerasimova’s method and found a different set of conditions that gave the same product in two steps as opposed to three as shown in Figure 1.19.

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**Figure 1.18** Synthesis of Nile red analogues with polyfluorinated ofefin substituent.

**Figure 1.19** Synthesis of polyfluorinated Nile red analogues.
Instead of starting with a completely fluorinated naphthalene, they opted to use the heptafluorinated analogue with a hydroxyl group at the 1-position. This was condensed directly with an already nitrosylated diethylaminophenol in dioxane. Products included both the $N$-oxide (in 37% yield as opposed to 3%) as well as a fully formed benzo[a]phenoxazinone dye. Reduction of the pot with zinc under acidic conditions converted remaining $N$-oxide to final dye and had a yield of 90%. Overall yield went from 0.5% to a much more acceptable 55%.

![Figure 1.20 Synthesis of polyfluorinated Nile red analogues in higher yield.](image)

1.6.7.2 Chlorinated Nile Blue Derivatives

The literature reports the preparation of some benzo[a]phenoxazinium dyes with chlorinated terminals. The syntheses of these compounds is relatively trivial and be accomplished as outlined by the general synthetic method shown in Scheme 1. Of most interest in the synthesis is the use of 1-bromo-3-chloropropane during the alkylation step. Bromine, being a better leaving group will have faster reaction kinetics than the chlorine terminal giving the corresponding chloropropyl substituents off of the amine. These are useful for conjugation to other molecules via simple $S_N2$ chemistry.
Figure 1.21 Benzo[a]phenoxazinium chlorides with chlorinated terminals.

1.6.7.3 Iodinated Nile Red Derivatives

Sherman et al. prepared Nile red analogues with iodo substituents for sensing thiols. Nucleophilic thiolate anions will attack the carbon vicinal to iodine in a facile $S_N2$ reaction. These dyes were created with the thiol-reactive iodine moiety extending off an alyl linker that was either attached via an oxygen at the dye’s 2-position or from the nitrogen at the 5-amino position as shown in Figure 1.22.

Dye 49 contains a primary alcohol that caps an alkyl substituted off of the 5-amino position. Stirring of 49 with iodoacetic anhydride in the presence of a strong organic base such as DMAP will give the substitution product 50 where the iodo-containing moiety is attached via an ester linkage.

To get the substitution off of the 2-position the 2-hydroxy Nile red (16) is used. Refluxing of 16 in DMF with $N$-bromoethylphthalimide and potassium carbonate will give dye 51. Addition of methylamine in MeOH to 51 followed by reflux will afford 52. The final reaction proceeds with 52 and iodoacetic anhydride in methylene chloride. No further base was added. This afforded dye 53 where the iodine-containing substituent was attached to the linker via an amide.
Figure 1.22 Synthesis of Nile red-based thiol sensors. A) Iodo sensor moiety is connected to 9-amino terminal via an ester linkage. B) Iodo sensor moiety is connected at 2-hydroxy position via an amide linkage.

1.6.8 Miscellaneous Syntheses

1.6.8.1 Bridged Naphthoxazinium Dyes

Kanitz and Hartmann prepared a novel set of benzo[a]phenoxazinium (and one phenoxazinium) salts. Naphthoxazinium salts are vulnerable to hydrolysis. By incorporating bridged systems either on the amine or on the naphthalene, it was hypothesized that hydrolysis could be prevented or at the very least, reduced. They incorporated a julolidine functionality where normally there is an $N,N$-dialkylated amine at the 9-position. One derivative contained a nitrogen heteroatom at the 4-position while another had sulfonamide group appended as a ring fused at the 4-carbon and 5-iminium nitrogen. They also prepared an alkylene-bridged benzophenoxazinium.$^{124}$ These are all highly novel and interesting structures in the benzo[a]phenoxazine literature.
1.6.8.2 Metalation of a benzo[a]phenoxazinone

Many dyes can chelate metals or have chelating appendages that will cause a dye to undergo optical shifts upon capture of a metal ion. Direct metalation of dyes is much rarer. The only examples of this in the literature were benzo[a]phenoxazines metalated directly with mercury (58).\textsuperscript{125, 126}

Mercuration of the Nile red core proved useful for synthesizing so-called FlAsH dyes (60) which use biarsenical ligands to selectively bind to cysteine as shown in Figure 1.24. Since Nile red reports on the local polar environment, they were able to use these dyes to monitor conformational changes in recombinant proteins in live cells upon addition of Ca\textsuperscript{2+}.\textsuperscript{125} Further work by Adams \textit{et al.} used biarsenical motif to monitor the formation of tetracysteine \textit{in vitro} and \textit{in vivo}.\textsuperscript{127}
Changing a carbonyl to either a cyano or dicyanomethylidene has been reported to shift absorbance wavelength bathochromically\(^{128, 129}\). For Nile red, this was an attractive option to modulate the scaffold for NIR applications. However, fluorescence no longer occurred even though the \(\lambda_{\text{max}}\) shifted from 550 nm to 643 nm\(^{105}\). Modification of the 5-oxo group was achieved using malononitrile, titanium tetrachloride and pyridine to afford 61 which has a dicyanomethylidene substituent in place of Nile red’s carbonyl. This is shown in Equation 3.

**Equation 3** Synthesis of Nile red analogue where the 5-oxo position has been substituted with a methylene dicyano moiety.

![Figure 1.24 Synthesis of Nile red FlAsH analogue.](image-url)
1.6.8.4 Replacement of 7-oxygen with a selenium atom

Foley et al. synthesized a short series of Nile blue analogues with varied chalcogen substitution (oxygen, sulfur and selenium) at the 7-position. They then ran a comparative analysis of all three as PDT agents against various bacterial strains to assess antibacterial efficacy. As expected, the selenium analogue outperformed the other candidates due to high triplet quantum yields.\textsuperscript{130} The data for this experiment was shown previously in Table 1.1.

As shown in Figure 1.25, synthesis started with \(\text{N,N-diethyl-3-iodoaniline} \) being mixed with magnesium turnings in dry ether to form the Grignard reagent. Dry selenium powder was added and allowed to react thoroughly. Then air was bubbled through the flask to give the diselenide product \(\text{63} \). Nitrosation of \(\text{63} \) was achieved the normal way as described in Scheme 1 to give \(\text{64} \) followed by condensation with \(\text{N-ethynaphthalen-1-amine} \) to give the final selenium dye \(\text{4} \).
Figure 1.25 Synthesis of Nile blue analogue with the oxygen replaced by selenium.

1.6.8.5 Microwave-assisted synthesis

Microwave heating allows for the rapid increase in temperature of a polar, bulk solvent. Since this kind of heating is caused by rapid polarization of the medium from the oscillating electromagnetic wave, the heat generated is generally uniform and the onset of high temperatures is fast. Conventional heating methods are slow due to a reliance on convection and thermal energy transfer through a glass reaction vessel. This makes the microwave an attractive option for rapidly completing reactions on a smaller scale.

Firmino et al. synthesized a series of Nile blue derivatives using conventional heating methods as shown in Figure 1.26. They then optimized the microwave synthesis of a model compound before proceeding with using the best conditions for the subsequent synthesis of their dye series. The best solvent was ethanol and high yields were achieved in three minutes or less of microwave irradiation.\textsuperscript{107}
Figure 1.26 Benzo[a]phenoxazinium chlorides 65a-f synthesized via microwave irradiation.

Reaction times using conventional heating were varied from as short as nine minutes to as long 280 minutes (over 4½ hours) as is shown in Table 1.2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Temp. [°C]</th>
<th>Time [min]</th>
<th>Yield [%][a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>65a</td>
<td>EtOH</td>
<td>65</td>
<td>9</td>
<td>96</td>
</tr>
<tr>
<td>65b</td>
<td>EtOH</td>
<td>65</td>
<td>280</td>
<td>89</td>
</tr>
<tr>
<td>65c</td>
<td>EtOH</td>
<td>65</td>
<td>50</td>
<td>77</td>
</tr>
<tr>
<td>65d</td>
<td>EtOH</td>
<td>65</td>
<td>155</td>
<td>34</td>
</tr>
<tr>
<td>65e</td>
<td>DMF</td>
<td>70</td>
<td>60</td>
<td>66</td>
</tr>
<tr>
<td>65f</td>
<td>EtOH</td>
<td>65</td>
<td>120</td>
<td>60</td>
</tr>
</tbody>
</table>

[a] Isolated yield.

Table 1.2 Synthesis of benzo[a]phenoxazinium chlorides 65a-f by using the conventional heating method.

For the microwave reaction solvent had to be optimized using a model system. Firmino used 65a as their model dye and irradiation with microwaves gave the best results—highest yields and shortest times—in ethanol as shown in Table 1.3.
<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temp. [°C]</th>
<th>Time [min]</th>
<th>Yield [%][a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>90</td>
<td>10</td>
<td>NR</td>
</tr>
<tr>
<td>MeCN</td>
<td>90/120</td>
<td>8/40</td>
<td>59</td>
</tr>
<tr>
<td>DMF</td>
<td>90</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>EtOH</td>
<td>90</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>EtOH</td>
<td>90</td>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td>H₂O</td>
<td>90</td>
<td>—</td>
<td>NR</td>
</tr>
</tbody>
</table>

[a] Isolated yield; NR: no reaction. [b] Solvent-free (silica support).

Table 1.3 Optimization reaction studies of nitrosophenol 27 with 1-amino-6-hydroxynaphthalene under microwave conditions

Using their previously established microwave conditions, all of their Nile blue analogues (65a-f) were synthesized again in three minutes or less with the lowest yield being 75% as shown in Table 1.4. These are remarkably efficient results and researchers hoping to synthesize phenoxazine dyes in the future would be wise to incorporate microwave synthesis into their protocols.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Time [min]</th>
<th>Yield [%][a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>65a</td>
<td>EtOH</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>65b</td>
<td>EtOH</td>
<td>1.3</td>
<td>93</td>
</tr>
<tr>
<td>65c</td>
<td>EtOH</td>
<td>1.3</td>
<td>90</td>
</tr>
<tr>
<td>65d</td>
<td>EtOH</td>
<td>2</td>
<td>86</td>
</tr>
<tr>
<td>65e</td>
<td>DMF</td>
<td>—</td>
<td>NR</td>
</tr>
<tr>
<td>65f</td>
<td>EtOH</td>
<td>2.5</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 1.4 Synthesis of benzo[a]phenoxazinium chlorides 65a-f by microwave irradiation at 90 °C.

1.6.8.6 Ultrasonic Synthesis

Sonication of a sample is another method of achieving rapid energy transfer into a reaction vessel. Pulses of ultrasonic radiation can generate large mechanical loads onto a molecule and in some cases can be strong enough to overcome the activation energy barrier required to initiate a reaction.
Ragu et al. reported synthesizing a series of Nile blue derivatives using ultrasound as shown in Figure 1.27. Reaction times for dye formation were three times faster (66b, 66c, 66f) up to as high as seven times faster (66g) as seen in Table 1.5. Even on the low end of reaction speed, the rates were still significantly higher. Product yields were barely affected except for 66b which showed a large increase in yield from 53% to 93%. For their conventional methods, they refluxed the reagents in ethanol/HCl.

![Figure 1.27 Dyes prepared via sonication](image)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Reaction Time (min)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional</td>
<td>US</td>
</tr>
<tr>
<td>6a</td>
<td>480</td>
<td>90</td>
</tr>
<tr>
<td>6b</td>
<td>290</td>
<td>90</td>
</tr>
<tr>
<td>6c</td>
<td>270</td>
<td>90</td>
</tr>
<tr>
<td>6d</td>
<td>480</td>
<td>105</td>
</tr>
<tr>
<td>6e</td>
<td>450</td>
<td>300</td>
</tr>
<tr>
<td>6f</td>
<td>180</td>
<td>64</td>
</tr>
<tr>
<td>6g</td>
<td>540</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 1.5 Reactions times and yields for the synthesis of dyes 66a-66g (Scheme 17) using conventional heating methods versus ultrasound (US).

### 1.7 Sensors

#### 1.7.1 Metal Sensors

Hu et al. developed a Nile blue-based chemodosimeter for Hg$^{2+}$ ions in aqueous solution. The Nile blue sensor undergoes a hypsochromic shift from 650 nm to 576 nm and has its fluo-
rescence quenched in the presence of Hg$^{2+}$ ions.\textsuperscript{132} The authors claim that the mercury-induced intramolecular reaction is what caused the quenching and that it was not complexation of the mercury ion with the chromophore. We dispute this claim as there is nothing about structure 68 that suggests it would not fluoresce. We propose that mercury is both causing the reaction and complexing with product to quench fluorescence and more evidence is needed to substantiate the authors’ claims.

As shown in Figure 1.28, compound 67 will capture the Hg$^{2+}$ ion with the sulfur atom. This activates the carbon vicinal to the sulfur for intramolecular nucleophilic attack from the 5-amino group. This intermediate will rearrange forming a five-membered ring and subsequently eliminates the sulfur-mercury complex to give 68.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure.png}
\caption{Proposed mechanism for mercury-induced rearrangement.}
\end{figure}

\textbf{1.7.2 pH Sensors}

Madsen \textit{et al.} developed a Nile blue-based pH sensor for \textit{in vivo} imaging.\textsuperscript{133} They cited an issue in the literature with a lack of effective probes for monitoring the pH in the interstitial fluid between cancer cells. Most probes would penetrate into the cell and report on the pH of intracellular fluid. They developed Nile blue labeled PMPC-PDPA diblock copolymers that would self-assemble into vesicles.
Nile blue is protonated at the 5-amino position. Deprotonation of this position in base will generate a neutral dye that has significantly different optical properties than its charged counterpart. This is because the charged iminium acceptor is changed to an amino donor. Without a donor-acceptor system, the ICT transfer band increases in energy giving a large hypsochromic shift.

As shown in Figure 1.29, Nile blue is mixed with methacrylic anhydride and triethylamine (TEA). TEA will deprotonated the 5-imino group, converting it into a nucleophile. This will attack the anhydride and eliminate the ester. Workup in dilute hydrochloric acid will regenerate the dye as the HCl salt 69 (the Nile blue methacrylamide or NBM). Dye 70 was created under identical conditions and workup but used a carbamate instead of an anhydride to give a urea linkage. This is the Nile blue 2-methacryloyloxyethyl carbamate or NBC.

![Synthesis of Nile blue-based comonomer](image)

**Figure 1.29** Synthesis of Nile blue-based comonomer. The PMPC-PDPA diblock copolymers were generated with a bromine on the terminal to function as a leaving group. As seen in Equation 4, Nile blue, NBM and NBC will all attack and substitute at this position to become fully conjugated to the polymer (72).
Equation 4 Synthesis of PMPC-PDPA diblock copolymers terminated with either Nile blue (NB) alone containing a Nile blue-based comonomer (either NBM or NBC).

1.7.3 Biological Sensors

1.7.3.1 Lysotracker

A Nile blue sensor with a morpholine substituent (NBM) was prepared by Fan et al. using the general synthetic method described in Figure 1.9. The morpholine acted as the targetable group that allowed the dye to bind to lysosomes. The morpholine substituent was substituted onto 1-aminonaphthalene using 4-(2-chloroethyl)morpholine and cesium carbonate as the base as shown in Figure 1.30.

Nile blue was chosen as an alternative to other probes due to its NIR absorption and emission profile, high extinction coefficient/quantum yield, and good stability against photobleaching. It was demonstrated that NBM was a viable lysotracker by using it to observe morphological changes to lysosomes during cell apoptosis as well as the production of lysosomes during cell division.
Another method for sensing hydrogen sulfide was investigated by Liu et al. They utilized a 2,4-dinitrobenzene moiety by conjugating it to the dye scaffold (either Nile red or Nile blue) in order to quench fluorescence. In the presence of hydrogen sulfide, the group is cleaved resulting in a return of fluorescence.\textsuperscript{135} The use of the 2,4-dinitrophenyl ether for sulfide sensing is relatively new to the chemical literature and had not been applied to a benzo[a]phenoxazine architecture.\textsuperscript{136,137,138}

As shown in Figure 1.31, the nitroso salt can be condensed with either an aminonaphthalene or a hydroxynapththalene as originally described in Scheme 1 to give dyes 75 or 77 respectively. Refluxing these dyes in DMF with 1-bromo-2,4-dinitrobenzene and potassium carbonate will cause the substitution of the dinitrophenyl group at the 3-hydroxy position. This quenching moiety is susceptible to attack from hydrogen sulfide.
**Figure 1.31** Synthesis of hydrogen sulfide detecting benzo[a]phenoxazine systems.
2 SYNTHESIS, OPTICAL CHARACTERIZATION AND BINDING STUDIES OF NOVEL BENZO[A]PHENOXAZINE DYES

2.1 Introduction

Popular dye structural motifs such as the BODIPY, squaraine, rhodamine and carbocyanine fluorophores have been explored exhaustively for their ability to act as visual reporters for a large variety of chemical processes and cross-platform applications.\textsuperscript{31-33, 35} Another classic dye structure, the benzophenoxazine, has attracted considerable interest in recent years. Benzophenoxazine dyes consist of a chemically robust, polycyclic aromatic core and are typically characterized by strong absorption of visible light and high fluorescence quantum yields in apolar media.

In the benzophenoxazine family of dyes, Nile red and Nile blue are popular due to their high fluorescence quantum yields and optical sensitivity to their local solvent environment. These compounds were first synthesized in 1896 by Möhlau and Uhlmann and have since been appropriated for a variety of histological applications.\textsuperscript{36, 75, 76, 106}

Nile red is a powerful solvatochromic and thermochromic reporter. It is highly lipophilic and as such it is poorly soluble in water. To rectify this problem, work has been done to append water soluble moieties to the dye scaffold. Solubilizing the dye in aqueous media causes optical spectra to undergo a 100 nm bathochromic shift into the near infrared spectrum.\textsuperscript{39, 139} Nile blue, is a cationic dye with much better water solubility than Nile red. It is highly susceptible to changes in pH and this makes it a great pH reporter. Nile blue has an iminium functional group that acts as a potent electron acceptor. Subsequent intramolecular charge transfer through the chromophore’s D-\pi-A system results in an 80 nm bathochromic shift in absorbance compared to Nile red.
Figure 2.1 Nile blue and Nile red dyes; convenient positions for structural modification are at the 2-carbon and 9-amine. The 5-imine on Nile blue is also an option for modification.

Since both the Nile red and Nile blue dye scaffolds have biologically useful NIR absorption bands when solvated in aqueous media, they could potentially be used for a variety of imaging applications. An imaging agent that offered inspiration for this project was oxazine-4, an amino-phenoxazineiminium dye containing secondary amino substituents. It showed a remarkable ability to target and bind to nerves.\textsuperscript{29} Benzo[a]phenoxazine dyes contain one more fused benzene ring than phenoxazine systems. By mimicking the secondary amine from oxazine-4, perhaps another nerve-binding agent can be constructed?

Before \textit{in vivo} work can be started, a comparative study of optical properties and protein—ligand affinities between Nile red and Nile blue derivatives must be undertaken. It was the aim of this study to synthesize analogous sets of Nile red and Nile blue derivatives with both tertiary and secondary amino substituents at the 9-position. Hydrophobicity was modified by appending alkyl groups of different sizes to the amine. Optical properties were investigated to understand how amino substituents modulate the chromophore. Human serum albumin (HSA), the most abundant transport protein in the blood was chosen as a model biological system. HSA—dye binding affinities were investigated and compared against calculated distribution coefficient values (log\textit{D}) to find viable dye candidates for \textit{in vivo} applications.\textsuperscript{†}

\textsuperscript{†} Analytical work with HSA was conducted by our collaborator Eman Alsolmy from the Patonay group.
2.2 Experimental Overview

2.2.1 General

All starting reagents used were obtained commercially from major chemical suppliers and were not purified before use. All $^1$H NMR and $^{13}$C NMR data were obtained on a Bruker Avance 400 spectrometer. HRMS data were obtained on a Waters Micromass Q-TOF mass spectrometer coupled with a Waters 2695 HPLC. Fatty acid free human serum albumin ($\geq 96\%$ purity) was obtained from Sigma-Aldrich (St. Louis, MO). DMSO was obtained from Acros Organics (99.9\%) (Belgium) and methanol was obtained from Sigma-Aldrich ($\geq 99.9\%$) (St. Louis, MO). Sodium phosphate monobasic and sodium phosphate dibasic were obtained from Aldrich Chemical Company (Milwaukee, WI). Absorbance spectra were acquired using a Cary 3G UV-visible spectrophotometer (Varian Inc., Palo Alto, CA) interfaced to a PC. Fluorescence spectra were achieved using a K2 Spectrofluorometer (ISS Inc., Champaign, IL) interfaced to a PC. Excitation was achieved using a Xenon Arc lamp, model PS 300-1, (ILC Technology Inc., Sunnyvale, CA). Spectroscopic studies were performed in disposable plastic cuvettes and binding studies were performed in quartz cuvettes, both with a path length of 1 cm. Nanopure water was obtained using an ELGA Purelab Classic water purification system.

2.2.2 Preparation of 3-amino-4-methylphenol

![Figure 2.2 Multi-route catalytic reduction of 4-methyl-3-nitrophenol to 3-amino-4-methylphenol.](image-url)
4-Methyl-3-nitrophenol (15 g, 98 mmol) was dissolved in 50 mL of MeOH. A catalytic amount of palladium on carbon was added. The flask was degassed and then put under hydrogen atmosphere three separate times to ensure the removal of oxygen. The reaction was run at room temperature for four days with hydrogen supplied via balloon. The balloon was changed several times over the course of the reaction. The reaction was followed by TLC until completion. Product (79) was filtered over a Celite pad in a Buchner funnel to remove catalyst. The filtrate was concentrated on a rotary evaporator to recover the product (95% yield.) No further purification was needed. A second route was also used for further synthesis of compound 79 as shown in Figure 2.2. Ammonium formate with palladium on carbon will transfer hydrogen to the molecule being reduced. This is most effective if refluxed in MeOH but care must be taken to keep the condensers clean. Ammonium formate will sublime and then deposit in the base of the condenser. This can cause blockage which, with the constant evolution of hydrogen gas, can cause dangerous complications if not taken care of promptly.

### 2.2.3 Preparation of 3-(dialkylamino)phenols and 3-(alkylamino)phenols

**Route 1**

\[
\text{H}_2\text{N} \quad \begin{array}{c}
\text{OH} \\
\end{array} \xrightarrow{\text{K}_2\text{CO}_3, \text{DMF}} \begin{array}{c}
\text{R} \\
\text{N} \\
\text{OH} \\
\text{R} \\
\end{array} + \begin{array}{c}
\text{R} \\
\text{N} \\
\text{OH} \\
\text{R} \\
\end{array}
\]

**Route 2**

\[
\text{H}_2\text{N} \quad \begin{array}{c}
\text{OH} \\
\end{array} \xrightarrow{\text{Kl, THF}} \begin{array}{c}
\text{R} \\
\text{N} \\
\text{OH} \\
\text{R} \\
\end{array} + \text{minor impurities}
\]

| a: R = Et | d: R = Et |
| b: R = Bu | e: R = Bu |
| c: R = PhPr | f: R = PhPr |

**Figure 2.3** Synthesis of N-alkylated 3-aminophenols
All of the alkylated 3-aminophenols (80a-f), with the exception of the 4-methyl substituted ones, were prepared by first dissolving 3-aminophenol in DMF. Stoichiometric amounts of finely powdered K$_2$CO$_3$ were added (1:1 for mono-substitution, 2:1 for di-substitution) and the system was mixed rapidly and heated to 50 °C. The alkyl halides were all liquids and were added dropwise. Reactions were monitored by TLC until completion. Compounds 81d-f were prepared in a similar manner but using dry THF as the solvent and using KI as a catalyst instead of base. Iodide is weakly nucleophilic and can act as a catalyst. Very little of the dialkylated products were formed using this procedure.

Reaction workup proceeded by first removing all solvent under reduced pressure. The products, which were all oils, were then taken up in DCM and filtered to remove salts. The reaction mixtures were all purified using column chromatography on normal phase silica and eluting with various compositions of EtOAc/Hexanes.

2.2.4 Synthesis of nitrosylated intermediates

Figure 2.4 Nitrosation of an N-alkylatedaminophenol.
All nitrosation reactions were conducted in the same manner as shown in Figure 2.4. An alkylated aminophenol was dissolved in EtOH or IPA and a molar excess of HCl was added. The entire system was cooled on an ice bath. Sodium nitrite (1.2 mol. eq.) was dissolved in 2 mL of DI water and very carefully added dropwise to the reaction flask over 15-20 min. The reaction was allowed to stir for an additional 2-3 h on the ice bath.

Solvent was removed under reduced pressure and the reaction residues were taken up in EtOH and filtered to remove inorganic salts. Nitroso products are unstable and were dried used in the next step without further purification. They were stored in a refrigerator when not being used.
2.2.5 Synthesis of benzo[a]phenoxazinone and benzo[a]phenoxazinium perchlorate dyes

Figure 2.5 Synthesis of Nile red and Nile blue derivatives

Dialkylated Nile red derivatives (84a-c) were prepared by dissolving the nitrosylated precursor in DMF with 1,6-dihydroxynaphthlene in 1:1 stoichiometric ratios. The solutions were heated to 90 °C for several hours. Dye formation was confirmed visually and the reactions were halted when UV-Vis spectra showed only the dye peak at 550 nm and the nitroso peak at 400 nm had completely disappeared. Monoalkylated Nile red derivatives (85d-f) were prepared the same way except using ethanol instead of DMF as a solvent and adding catalytic amounts of HCl. All
Nile blue derivatives (86a-f) could be prepared by refluxing the nitroso and aminonaphthalene precursors in ethanol with perchloric acid.

2.2.6 Spectroscopic studies of dyes

2.2.6.1 Determination of molar absorptivity

Serial dilutions of each dye were prepared in DMSO and methanol with concentrations ranging from 0.2×10^-5 – 1.60×10^-5 M. It was achievable to determine the molar absorptivity values in 20 mM phosphate buffer pH 7.2 for some monoalkylated dyes specifically 86a and 86b in the same concentration range. All working solutions in methanol and phosphate buffer contained only 1.6% (v/v) DMSO to facilitate dye dissolution. The maximum wavelength of absorption (λ\text{Abs}) of each dye was determined. The molar absorptivity values of each dye were determined using Beer’s law. Duplicate absorbance measurements were obtained, and the average molar attenuation values were reported.

2.2.6.2 Determination of quantum yield

Serial dilutions of each dye were prepared in DMSO and methanol with concentrations ranging from 0.2×10^-6 – 1.60×10^-6 M, at concentrations diluted tenfold more than the absorbance measurements in which the absorbance values were below 0.1 units to avoid inner filter effects or self-quenching. Quantum yields values were determined in 20 mM phosphate buffer at pH 7.2 for some monoalkylated dyes, particularly Nile blue derivatives 86d and 86e in the same concentration range. All working solutions in methanol and phosphate buffer contained only 1.6% (v/v) DMSO to facilitate dye dissolution. The relative method was used to determine quantum yield values for all dyes using Nile blue as a standard and applying the equation:

\[
\Phi_S = \Phi_S \left[ \frac{\text{Slope}_S}{\text{Slope}_{\text{STD}}} \right] \left[ \frac{\eta^2_{\text{STD}}}{\eta^2_{\text{STD}}} \right]
\]
Where $\Phi_S$ and $\Phi_{STD}$ are the quantum yield of the sample and the standard, slope $S$ and slope $STD$ are the slopes of plotting the area under the emission curve as a function of the absorbance value at excited wavelength of the sample and the standard. $\eta_S$ and $\eta_{STD}$ are the refractive index of solvent of the sample and the standard. Duplicate measurements were obtained, and the average quantum yield values were reported. All solutions in DMSO were excited at 580 nm and in methanol and phosphate buffer were excited at 560 nm. The excitation wavelengths were chosen to ensure the full integration of emission peaks, especially for the monoalkylated derivatives since they are blue shifted compared to dialkylated derivatives.

2.2.7 **HSA binding interaction with Nile derivatives**

The initial investigation of binding interactions between HSA and the dye involves using fluorescence spectroscopy to monitor a fixed concentration of the dye itself in buffer first. Then the same concentration of the dye is mixed with a similar concentration of the protein assuming 1:1 stoichiometry in buffer. The dye concentration is determined by which the absorbance value should be below 0.1 units to avoid inner filter effects or self-quenching. Using fluorescence spectroscopy allows for observing any changes that might occur in the maximum wavelength of emission (bathchromic or hypsochromic shift) or in fluorescence intensity (hyperchromic or hypochromic shift) after interactions between the dye and the protein. For Nile blue derivatives, $2.0 \times 10^{-6}$ of the dye in 20 mM phosphate buffer pH 7.2 was recorded first. Then, $2.0 \times 10^{-6}$ of the dye with $2.0 \times 10^{-6}$ of HSA in 20 mM phosphate buffer pH 7.2 was recorded. To confirm the HSA—dye binding ratio, Job’s method was used to identify the stoichiometry as discussed in the following section.
2.2.7.1 *Determination HSA-dye stoichiometry using Job’s method*

Job’s method or the method of continuous variation is used to determine the stoichiometry of the predominant complex when multiple interactions can occur. This method includes varying the number of moles for the protein and the dye whereas the total concentration of the solution remains constant. Fluorescence spectroscopy can be used to identify this predominant complex which is corresponding to maximum fluorescence intensity as a function of the mole fraction of HSA. Thus, variation of the number of HSA moles resulted in a maximum mole fraction of 0.50 for the dialkylated dyes (NB, 86a, 86b, and 86c) complexes, which corresponded to 1:1 stoichiometry of HSA—dye complex. For the monoalkylated dyes, Job’ method provides another evidence that these dyes did not bind to HSA. The total concentration of HSA—dye complex was 4.0×10⁻⁶ M.

2.2.7.2 *Effect of time on HSA—Dye complex*

The effect of time on solution containing 2.0×10⁻⁶ M of dye and 2.0×10⁻⁶ M of HSA was studied in 20 mM phosphate buffer pH 7.2. The reaction was monitored by recording the fluorescence intensity every 5 minutes for 30 minutes at room temperature.

2.2.7.3 *Determination binding constant using Scatchard plot*

The HSA binding interaction with NB, 86a, 86b and 86c were studied by titrating a fixed concentration of dye (2.0×10⁻⁶ M) with different micromolar concentrations of HSA in 20 mM phosphate buffer pH 7.2. All working solutions contained only 1% (v/v) DMSO to facilitate dye dissolution and more importantly to avoid HSA denaturation. The HSA—dye complex was vortexed for 30 seconds and then left five minutes to allow enough time for it to reach equilibrium. Duplicate measurements were obtained, and the average binding constant values were reported. To determine the binding constant of the HSA—dye complex, the complex formation was evalu-
ated using Scatchard’s method. The concentrations of the different reaction species were measured at equilibrium. Applying the equation,

\[
\text{Dye + HSA} \rightleftharpoons \text{Dye-HSA}
\]

and assuming 1:1 reaction stoichiometry

\[
K = \frac{[\text{Dye-HSA}]}{[\text{Dye}][\text{Dye}]}
\]

the binding constant (K) is determined from the linear fit of the data of the fluorimetric titration of a fixed concentration of dye with HSA according to equation:

\[
\frac{1}{\Delta F} = \frac{1}{k[Dye]} + \left(\frac{1}{k[Dye]K}\right) \frac{1}{[\text{HSA}]}
\]

Where \(\Delta F\) represents the change in emission intensity of the HSA—dye complex, \(k\) is a constant dependent on the quantum efficiency of the process and the instrumentation. A plot of the reciprocal of \(\Delta F\) versus the reciprocal of HSA concentration gives a straight line relationship that intersects the x-axis. The binding constant is calculated by dividing the intercept by the slope of the line.

2.3 Results and Discussion

2.3.1 Synthesis of Nile Red Analogues 84a-c and 85d-f.

The syntheses of the Nile red analogues 84a-c and 85d-f are illustrated in Figure 2.5. The starting materials used for the synthesis depended on the degree that the 9-amino position was to be substituted. For the tertiary amine-containing dyes, the first reaction was an alkylation of 3-aminophenol. Alkylating agents used were iodobutane and 1-bromo-3-phenylpropane. 3-\((N,N\)-diethylamino)phenol was available commercially. Alkylations were conducted in DMF at 70 °C in 2:2:1 molar equivalents of potassium carbonate, alkylating agent, and 3-aminophenol respec-
tively. After TLC showed full consumption of the starting reagents, workup of the mixture consisted of first removing all solvent under reduced pressure. The oily residues were then taken up in ethanol, filtered to remove salts, and then loaded onto silica for purification. All of these alkylated aminophenols were purified on columns using normal phase silica and ethyl acetate/hexanes solvent systems.

The next step was nitrosation of the alkylated amino phenols para to the amino position via Fischer-Hepp rearrangement. The starting material was dissolved in ethanol and concentrated HCl was added carefully. The system was then cooled on an ice bath. Sodium nitrite (1.2 mol. eq.) was dissolved in water and added slowly over 20-30 min. The reaction was kept stirring and cold for 3 hours afterwards. Solvent was then removed under reduced pressure until all water was pulled off with special care taken not to heat the system over 50 °C. Overheating would decompose the nitroso compound and the contents of the flask would turn first green, then blue. It is most likely that the nitroso salts react with each other at elevated temperatures to form some sort of azo dye. The compound was then taken up in ethanol and filtered to remove salts. Solvent was removed again under reduced pressure to yield solid, yellow nitroso hydrochloride salts.

Dye formation was achieved via a condensation reaction between the nitroso salts and 1,6-dihydroxynaphthalene in a 1:1 mole ratio. Reactants were heated to 90 °C in DMF until UV-Vis showed the disappearance of the nitroso peak (~400 nm). DMF was then removed under reduced pressure and the dye was taken up in a small volume of ethanol. Solubility of the dyes was sparing in this solvent. Vacuum filtration of the mixture gave spectroscopically clean dyes that needed no further purification.

Synthesis of Nile red analogues with a secondary amine presented a different challenge because alkylation of 3-aminophenol would be driven to higher degrees of substitution forming ter-
tary and quaternary amines/ammonium salts. To combat this, 4-methyl-3-aminophenol was used where the methyl group would act as a steric blocker, slowing down kinetics for subsequent, unwanted alkylations. This compound was prohibitively expensive so 3-nitroaminophenol was purchased and then reduced to the corresponding amine using palladium on carbon and hydrogen gas.

In order to further protect the amine, alkylations were conducted in THF using catalytic amounts of potassium iodide and one molar equivalent of alkylating agent to aminophenol. Workup and purification as well as nitrosation were conducted in the same fashion as previously stated.

Dye formation was achieved as previously stated except that ethanol was used instead of DMF and catalytic amounts of HCl were added. The reaction mixture was refluxed until complete as verified by UV-Vis spectroscopy. Workup and purification were the same as for the previous dyes.

2.3.2 Synthesis of Nile Blue Analogues 86a-f.

Compounds 82a-f described previously were used to make 86a-f. Condensation of 1-aminonaphthalene with 82a-f were carried out in ethanol and slight molar excess of perchloric acid in a sealed tube at 90 °C. Compounds 86a-f are only slightly soluble in cold ethanol and can simply be filtered to yield a product of high purity.
2.3.3 Proposed mechanism of dye formation.

Figure 2.6 Proposed mechanism for the formation of Nile red via condensation of an aromatic nitroso compound with 1,6-dihydroxynaphthalene in catalytic amounts of acid.

Dye formation is the product of an acid-catalyzed condensation reaction between a nitroso precursor and 1,6-dihydroxynaphthalene or 1-aminonaphthalene. A mechanism for this reaction has been proposed. The first step is nucleophilic attack by the carbon para to the 1-hydroxy position on the dihydroxynaphthalene to the nitrogen on the nitroso group. The oxygen on the nitroso group resembles a carbonyl with its double bond and should make the nitrogen more electrophilic. After the attack, the electrons in the double bond will move to the oxygen and will readily receive a proton from an acid.
The resulting intermediate is no longer aromatic and is carrying a positive charge on the 1-hydroxy position. Intramolecular proton transfer from the previously nucleophilic carbon to the N-hydroxyl group will allow restoration of aromaticity. The diethylamine will donate electrons into the conjugated system causing water to leave what had been the nitroso nitrogen.

The enol on the right side of the molecule can be deprotonated by water and will tautomerize to a ketone. The cascading electron push will neutralize the positive charge on the diethylamine. The ketone is very similar to an $\alpha,\beta$-unsaturated system and should cause the $\beta$ carbon to act as a Michael acceptor. The Michael donor will be the phenolic oxygen on the adjacent aromatic ring. The lone pair on this oxygen will attack the electron deficient $\beta$ carbon and then be deprotonated by water.

The enolate that formed on the right side of the molecule will pick up a proton from acid. This will set up the electronics to favor deprotonation by water at the $\beta$ carbon where the oxygen had previously attacked. As the electrons push towards the enol, the oxygen will gain a positive charge allowing for water to act as a base and abstract a proton from this position. This step will complete the formation of the neutral dye molecule (for Nile red).

2.3.4 Spectroscopic Properties of Nile red and Nile blue analogues.

The optical properties of all Nile red and Nile blue derivatives were conducted in both DMSO and MeOH. In DMSO, monosubstitution at the 9-amino position of the benzo[a]phenoxazinone dye scaffold showed several noticeable trends. Absorbance shifted by 3-6 nm hypsochromically and emission by 8-9 nm hypsochromically when going from a tertiary amine to a secondary amine for Nile red derivatives. Stokes shift reduced by 3-7 nm while the quantum yield showed almost no difference using both standards (R6G and RB). Since the disubstituted dyes had an extra alky substituent, it would have more vibrational and rotational modes. These extra modes,
when summed across the entire molecule, should be responsible for greater loss of energy from $S_1$ via internal conversion and this is apparent because the disubstituted Nile red analogues have larger Stokes shifts.

<table>
<thead>
<tr>
<th>dye</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Phi_f^*$</th>
<th>fwhm (nm)</th>
<th>solvent</th>
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<tr>
<td>NR</td>
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<td>40,300</td>
<td>633</td>
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<tr>
<td></td>
<td>554</td>
<td>39,100</td>
<td>631</td>
<td>0.28</td>
<td>61</td>
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</tr>
<tr>
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<td>32,500</td>
<td>627</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>72,700</td>
<td>634</td>
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</table>

* $\Phi_f$-R6G = 0.95 and $\Phi_f$-RB = 0.70 in EtOH

**Table 2.1** Optical properties of Nile red and Nile blue analogues
In MeOH, monosubstitution at the 9-amino position of the dye scaffold showed similar trends to the dyes with dissubstitution of the 9-amino position, but they diverged very strongly in quantum yield. Absorbance shifted by ~5 nm hypsochromically and emission by 12-14 nm hypsochromically when going from a tertiary amine to a secondary amine. Stokes shift reduced by 6-8 nm while quantum yield showed a marked increase by 10-13% across the board for derivatives with secondary amines.

Nile blue derivatives showed similar optical trends to Nile red with a few notable exceptions. Monosubstituted varieties of Nile blue had very high quantum yields and these increased significantly when measured in MeOH as opposed to DMSO. This is the opposite as was observed for Nile red derivatives. Here we see a polar, protic solvent increasing fluorescence output. Why is that? One proposed reason is that Nile blue has many more hydrogen bond donors than Nile red does. Perhaps a hydrogen bond matrix formed between dye and solvent can work to increase the local viscosity. This will of course have the expected outcome of increasing fluorescence quantum yields.

Now that optical properties had been determined in organic media, our attention was turned to how the dyes responded in aqueous, buffered environments as shown in Figure 2.8.
The Nile red analogues were dissolved in 30 mM phosphate buffer (pH = 7.2) to make 30 μM stock solutions. The absorbance spectra of each of the dyes showed differing $\lambda_{\text{max}}$ as well as varying absorption profiles. This was interesting because $\lambda_{\text{max}}$ was largely unchanged in MeOH and DMSO from dye to dye. It suggested that the pK$_a$ of each dye species can be substantially modified by its substituents and degree of substitution. Nile red and dye 84a had the same broad shape but had slightly different absorption intensities. 84a only has an extra hydroxyl group from Nile red so they can be expected to be similar. 84b and 84c were practically identical in both line shape and intensity in their respective UV-Vis spectra. Both the butyl and phenylpropyl substituents were very hydrophobic and both dyes contained tertiary amines so similar effects were not surprising. 85d and 85e which have N-ethyl and N-butyl substitutions, respectively, both gave a yellow color with 85e having weaker intensity.

Dyes that contained tertiary amines had very little perturbation of $\lambda_{\text{max}}$ and minor differences in absorption intensity. If the dyes had a secondary amine then the immediate effect was to have a hypsochromic shift. The ethyl and butyl containing monsubstituted dyes shifted hypsochromical-
ly by approximately 100 nm. This was in opposition to 85f with a phenylpropyl group, which had a 20 nm hypsochromic shift.

A hypsochromic shift is indicative of a less conjugated system or if the donor/acceptor pair was modified to have a weaker push/pull effect across the conjugated pi system. Protonation of the secondary amine would give the molecule not one, but two effective acceptor moieties. Without the donor, excitation of the dye with light would require greater energies. It can be concluded that the monosubstituted dyes were being protonated effectively in buffer and that their pKₐs were subsequently higher than their analogous disubstituted analogues which had optical properties that were relatively unperturbed in buffer solution. Increasing substitution on an amine will increase its basicity.¹⁴¹ What is remarkable about this data is that it would appear that the less substituted amines were more basic than their tertiary counterparts.

The Nile blue derivatives showed less differentiation than Nile red dyes but the very hydrophobic 86c and 86f had very flat, broad absorption profiles. This is almost certainly due to poor solubility and aggregation.

**Figure 2.8** Normalized absorbance and fluorescence of 86d in phosphate buffer

86d and 86e showed nearly identical absorbance/fluorescence wavelengths in phosphate buffer. They were chosen from the Nile blue derivatives for having superior absorption in buffer. A
small shoulder present at 577 nm on the absorbance spectra (Figure 2.9) is absent from the fluorescence spectra. This is most likely an H aggregate band.

Now that dye performance and behavior in buffered aqueous media had been assessed, the project moved forward into investigating how the dyes would behave in a model biological system. Human serum albumin (HSA) was chosen as that system.

HSA is the most common protein found in human blood plasma. Because of how ubiquitous it is in the blood and because of its physiological mobility, it acts as a transporter for an astounding variety of small molecules. Whether or not a drug candidate binds to HSA and the strength of said binding interaction can have a pronounced effect on drug efficacy, retention/clearance, and toxicity.

A series of binding studies were done to see how the Nile red analogues bound to HSA. Fluorescence spectroscopy was used to determine binding constants. Nile red has a very low quantum yield in water. This is mostly due to aggregation caused by poor solubility.

**Figure 2.9** Fluorescence spectra of Nile red and Nile blue (5 μM) with HSA (5 μM) in 20 mM phosphate buffer pH = 7.2

As shown in Figure 2.9, Both Nile red and Nile blue showed significant increases in fluorescence upon binding to HSA. The dyes are very hydrophobic and flat so that they will tend to pi
stack and aggregate. The dyes should be prone to binding at hydrophobic sites on protein surfaces and grooves. Such binding would break up aggregates giving a large increase in observed fluorescence. Fluorescence increase could also be due to immobilization of dye molecules and restriction of rotation. Dyes in solution are constantly tumbling and there are many degrees of movement that deactivate the excited state. Immobilized on a protein, there are far less degrees of movement/rotation and the excited state is less likely to be perturbed. Now the binding of the Nile red and Nile blue derivatives had to be assessed.

**Figure 2.10** Fluorescence spectra of Nile red derivatives (5 μM) with HSA (5 μM) in 20 mM phosphate buffer pH = 7.2

The largest increase in fluorescence intensity was actually observed on Nile red with a 350 fold increase in Figure 2.9. Dye 84a decreased in fluorescence intensity and had a hypsochromic shift. This wavelength shift in and of itself could prove somewhat useful. 85d performed poorly because baseline fluorescence from the unbound dye was already present and the binding in-
crease was not substantially greater. 84b, 84c, and 85f all showed very little baseline fluorescence with large increases in fluorescence intensity upon binding with HSA as shown in Figure 2.10.

![Fluorescence spectra of Nile blue derivatives (5 μM) with HSA (5 μM) in 20 mM phosphate buffer pH = 7.2](image)

**Figure 2.11** Fluorescence spectra of Nile blue derivatives (5 μM) with HSA (5 μM) in 20 mM phosphate buffer pH = 7.2

Nile blue derivatives behaved quite differently with HSA than their Nile red counterparts. Only the disubstituted dyes (86a-c) showed any kind of fluorescence increase upon addition to HSA as seen in Figure 2.11. This would indicate that monosubstituted dyes 86d-f do not bind HSA. Of the disubstituted dyes, 86b and 86c had the greatest relative fluorescence increase from unbound versus bound. However, 86c had a low overall fluorescence intensity, especially compared to 86b which would appear to be the best dye.
Figure 2.12 Binding interactions of Nile red derivatives observed with change in fluorescence vs. time. Binding interactions of Nile blue derivatives observed with change in fluorescence vs. time.

Nile red, 84b, 85d, 85e, and 85f all had fast binding interactions that gave high fluorescence intensity as shown in Figure 2.12. Nile red, 84b, and 85f maintained the same intensities for 30 minutes. 85d and 85e lost intensity over time suggesting decomposition or dissociation from the dye—HSA complex. 84c showed increased binding over time as fluorescence intensity gradually increased, reaching its maximum after 20 minutes.

The next step was to determine the stoichiometry of the dye—HSA complex. This was achieved using Job’s method. Dye and HSA were mixed together at a fixed overall concentration. The mole fraction of the dye versus the HSA would be changed but the solution would be maintained at a constant concentration. The effect of this is that as dye binds to HSA, fluorescence increases. At high concentrations of dye, fluorescence will be low because there will not be enough protein in solution to bind all of it. As dye concentration decreases and protein concentration increases the fluorescence should increase. Eventually there will be more protein than dye to bind it. The mole fraction at which the apex of fluorescence intensity is reached is indicative of dye—protein binding stoichiometry.
Figure 2.13 Job’s plots of 84b (A), 84c (B), 86b (C) and 86c (D) with HSA. Overall concentration is 4 μM in 20 mM phosphate buffer pH = 7.2.

Out of all of the Nile red/blue derivatives, those shown in Figure 2.13 gave the best shapes for a Job’s plot which is that of an arrow. Monosubstituted Nile blue dyes gave completely linear Job’s plots suggesting that they did not bind at all to HSA. All of the plots shown showed maximum fluorescence at a mole fraction of 0.5 meaning that dye—HSA binding stoichiometry is 1:1.

Calculating logD values and their implications. The calculated distribution coefficient (logD) is a useful bioavailability predictor and its calculation is trivial using modern software. Chemaxon has logD calculator plugin in Marvin that is convenient to use. The calculated partition coefficient (logP) is the ratio of the concentration of a compound in n-octanol vs. water. It is
a parameter that clearly indicates the hydrophobicity of the target compound and can be used to screen molecules for biocompatibility before wasting resources on actual biological testing. The reason logD is preferred over logP is because it is more accurate as it takes into account both ionized and un-ionized forms of the target molecule while logP is much more one-dimensional, taking into account only the un-ionized form.

Low logD values indicate a molecule that is very hydrophilic. These molecules will typically be poor binders to transport proteins and can be rapidly removed via renal clearance. A logD value that is high is indicative of hydrophobicity. These compounds are prone to aggregate and/or bind irreversibly to proteins. They can persist in the body and this can cause toxic side effects and poor clearance.

The logD values of all of the dyes are displayed in Table 2.2. Compounds 84c and 86c were the most hydrophobic with logD values exceeding 4. Compounds 84b, 85f, and 86b were within the 2-4 logD value range while 84a, 85d, 85e, 86a, 86d, 86e, and 86f were less than 2.

Binding constants were determined using Scatchard’s method. The values did not correspond with any linearity or regularity to logD values. This is a good indication that hydrophobicity is not the sole determinant in binding affinity between the dyes and the protein.
<table>
<thead>
<tr>
<th>Dye</th>
<th>K (M(^{-1}))</th>
<th>clogD (pH =7.4)</th>
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<tbody>
<tr>
<td>NR</td>
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<td>84c</td>
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</tr>
<tr>
<td>85e</td>
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<tr>
<td>85f</td>
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<tr>
<td>86f</td>
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<td>1.71</td>
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\([a]\) Nile blue chloride salt

Table 2.2 HSA binding constants and clog\(D\) values of Nile red and Nile blue analogues.
3 CONCLUSIONS

A series of Nile red and Nile blue derivatives with increasing lipophilicity were synthesized. Alkylation of the 9-amino position was controlled to give dyes with either tertiary amines or secondary amines. Dyes with tertiary amines had higher extinction coefficients and larger Stokes shifts than dye with secondary amines. However, the secondary amine-containing dyes had noticeably larger quantum yields. Nile red with one ethyl substituent at the 9-amino position had the highest binding affinity to HSA out of all dye candidates. Nile blue differed with disubstituted candidates binding strongly and their monosubstituted counterparts hardly binding at all. The largest fluorescence increase from dye binding to HSA was observed with compound 86b which is a Nile blue derivative containing a tertiary amine with two butyl groups.
4 EXPERIMENTAL

4-methyl-3-aminophenol. The starting reagent, 4-methyl-3-nitrophenol (15 g) was dissolved put into a 250 mL round bottom flask and dissolved in 50 mL of MeOH. A catalytic amount of palladium on carbon was added. The flask was degassed and then put under hydrogen atmosphere three separate times to ensure the removal of oxygen. The reaction was run for four days with hydrogen supplied via balloon. The balloon was changed several times over the course of the reaction. The reaction was followed by TLC until completion. Product was filtered over a Celite pad in a glass sintered funnel to remove catalyst. The pad was washed with ethyl acetate and the filtrate was concentrated on a rotary evaporator to recover the product (91% yield.) No purification was needed. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.61 (s, 1H), 6.65 (d, 1H, $J = \text{Hz}$), 6.05 (d, 1H, $J = 4\text{Hz}$), 5.89 (q, 1H, $J = \text{Hz}$), 4.64 (s, 2H), 1.92 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 155.58, 146.69, 129.71, 111.20, 102.85, 100.61, 16.02

3-(dibutylamino)phenol. Iodobutane (4.17 mL, 3.67 mol) was combined with 3-aminophenol (2.00 g, 1.83 mol) in a 100 mL round bottom flask. Two equivalents of potassium carbonate were dissolved in a water/isopropanol solution which was then added to the reaction flask. The mixture was refluxed at 70 °C for 2 h. 55% yield.

3-(bis(3-phenylpropyl)amino)phenol. 3-aminophenol (2.00 g) was dissolved in DMF along with 5.06 g of $\text{K}_2\text{CO}_3$. The mixture was sonicated and allowed to mix for 15 minutes. Two molar equivalents (with respect to 3-aminophenol) of 1-bromo-3-phenylpropane (3.56 mL) were added dropwise at room temperature. The mixture was left to stir at room temperature for an additional 15 min and then was gradually heated to a maximum temperature of 70 °C over the course of two hours. TLC confirmed that all the starting material had been consumed but that three products had formed. The products were separated via regular phase column chromatog-
raphy eluting first with 1:1 v/v of Hexanes/DCM. This was increased to 100% DCM to finish the separation. The middle spot was confirmed by spectroscopic analysis of the eluting products to be the desired compound. 88% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.86 (s, 2H), 7.28 (m, 6H), 6.81 (t, 1H, J = 4Hz), 5.96 (m, 3H), 5.46 (s, 1H), 2.93 (s, 2H), 2.66 (t, 2H, J = 4Hz), 1.82 (t, 2H, J = 4Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 157.63, 149.82, 141.42, 128.90, 127.77, 127.76, 125.18, 103.01, 102.48, 98.28, 41.87, 32.23, 30.04.

3-(ethylamino)phenol. 3-aminophenol (4.00 g, 36.7 mmol), potassium carbonate (5.07 g, 36.7 mmol), and ethyl iodide (2.95 mL, 36.7 mmol) were combined in a flask with 20 mL of DMF. The reaction mixture was stirred and heated for 2 h at 100 °C. It was then concentrated under reduced pressure to remove all DMF. The residual oil was loaded onto a regular phase column and eluted with a 1:4 EtOAc/Hexanes mobile phase which was removed under reduced pressure. The product was a light brown oil and was recovered with a 51% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.13 (t, 3H, J = 8Hz), 2.96 (q, 2H, J = 8Hz), 5.99 (m, 3H), 6.83 (t, 1H, J = 8Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 14.36, 37.35, 98.89, 103.09, 103.59, 129.37, 150.18, 158.10.

3-(butylamino)phenol. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.002 (t, 1H, J = Hz), 6.194 (dd, 2H, J = Hz), 6.081 (s, 1H, J = Hz), 3.062 (t, 2H, J = 7.2 Hz), 1.579 (quin, 2H, J = 7.2 Hz), 1.407 (sex, 2H, J = 7.2 Hz), 0.944 (t, 3H, J = 7.2 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 156.73, 150.10, 130.17, 105.99, 104.26, 99.64, 43.74, 31.58, 20.29, 13.92; m.p. 59-60 °C

3-((3-phenylpropyl)amino)phenol. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.895 (s, 1H), 7.308-7.163 (m, 5H), 6.836 (t, 1H, J = 8.0 Hz), 6.024-5.963 (m, 3H), 5.464 (s, 1H), 2.959-2.948 (m, 2H), 2.672 (t, 2H, J = 7.6 Hz), 1.829 (quin, 2H, J = 7.6 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$
158.64, 150.82, 142.41, 129.91, 128.77, 128.74, 126.16, 104.01, 103.50, 99.30, 42.88, 33.23, 31.04; m.p. 101-103 °C

3-(ethylamino)-4-methylphenol. Compound 79 (3.85 g, 31.26 mmol) and iodoethane (0.5 mL, 6.25 mmol) were dissolved in anhydrous THF. A catalytic amount of KI (0.05 mol eq.) was added to the mixture. The reaction mixture was heated 50 °C for 5 h. TLC eluting with 4:1 hexanes/EtOAc showed only two spots. The top spot was the desired product and the bottom spot was the starting material which had been used in excess. The product was purified by normal phase column chromatography eluting with same mobile phase used in the TLC analysis. The product was a yellow oil. A solid was obtained by placing the product under high vacuum for several hours. The crystals were off white. Percent yield was 53% off of the column. $^1$H NMR (400 MHz, DMSO-$d_6$) 6 1.18 (t, 3H, J = 8Hz), 1.95 (s, 3H), 3.02 (t, 2H, J = 8Hz), 4.50 (s, 1H), 5.92 (d, 1H, J = 8Hz), 5.97 (s, 1H), 6.70 (d, 1H, J = 8Hz), 8.71 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) 6 14.38, 16.69, 37.42, 97.01, 102.11, 112.11, 129.93, 147.32, 156.46; m.p. 88-90 °C.

3-(butylamino)-4-methylphenol. Compound 79 (7.39 g, 60 mmol) and iodobutane (1.39 mL, 13.6 mmol) were dissolved in anhydrous THF. A catalytic amount of KI (0.05 mol eq.) was added to the mixture. The reaction mixture was heated 50 °C for 24 h. TLC eluting with 4:1 hexanes/EtOAc showed only two spots. The top spot was the desired product and the bottom spot was the starting material which had been used in excess. The product was purified by normal phase column chromatography eluting with same mobile phase used in the TLC analysis. The product was a yellow oil with an orange tinge. A solid was obtained by placing the product under high vacuum for several hours. The crystals were off white. $^1$H NMR (400 MHz, CDCl$_3$) 6 1.00 (t, 3H, J = 8Hz), 1.47 (sex, 2H, J = 8 Hz), 1.67 (quin, 2H, J = 8Hz), 2.08 (s, 3H), 3.14 (t, 2H, J =
8Hz), 6.12 (d, 1H, J = 4Hz), 6.16 (s, 1H), 6.90 (d, 1H, J = 8Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 13.83, 16.51, 20.24, 31.44, 43.71, 98.06, 103.33, 114.12, 130.47, 147.32, 155.12; MS (ESI): 179.6 (M$^+$); m.p. 47-49 °C.

**4-methyl-3-((3-phenylpropyl)amino)phenol.** Compound 79 (40 mmol) and 1-bromo-3-phenylpropane (1.23 mL, 8.1 mmol) were dissolved in anhydrous THF. The molar ratio of 79 to alkyl halide was 5:1. A catalytic amount of KI was added (0.5 molar eq.) The reaction mixture was heated to 45 °C for 48 h. TLC showed that some alkyl halide still remained but the reaction was halted at this time. The crude mixture was concentrated under reduced pressure and purified via normal phase chromatography eluting with 4:1 hexanes/EtOAc to get the product off of the column. Starting material 79 was easily recovered by then eluting with 100% EtOAc. The product was concentrated under reduced pressure to yield a viscous, yellow oil. The addition of 0.75 mL of CDCl$_3$ caused the oil to immediately crystallize. The crystal were further dried under vacuum and appeared an off white color. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.95 (t, 2H, J = 8Hz), 2.02 (s, 3H), 2.74 (d, 2H, J = 8Hz), 3.14 (t, 2H, J = 4Hz), 6.08 (d, 1H, J = 4Hz), 6.10 (s, 1H), 6.83 (d, 1H, J = 4Hz), 7.28 (m, 5H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 15.03, 29.76, 32.13, 42.20, 96.40, 101.72, 112.56, 116.43, 124.87, 127.46, 129.43, 141.38, 146.55, 155.39; MS (ESI): 241.4 (M$^+$); m.p. 95-96 °C.

**5-(diethylamino)-2-nitrosophenol hydrochloride.** A solution of 3-(diethylamino)phenol (1.07 g, 6.48 mmol) was prepared by dissolving it in HCl (8 mL, 32%) and 20 g of ice. The solution was kept at 0-5 °C. A solution of sodium nitrite (0.4471 g, 6.48 mmol) and DI water (6 mL) was added drop wise to previous solution for 1 h at 0-5 °C. The resulting brown slurry was stirred for 2 h at 0-5 °C. The slurry was filtered and the filtrate was collected and dried under re-
duced pressure. Due to the unstable nature of reported nitroso compounds the product was used without any further purification.100

5-(dibutylamino)-2-nitrosophenol. Compound 80b was dissolved in 10 mL of isopropanol. HCl (8 ml, 32%) was added to the solution along with 20 g of ice. The solution was kept on an ice bath, stirring for the remainder of the reaction to maintain a temperature at 0-5 °C. Over a period of 1 h, an 8 mL of solution of sodium nitrite (0.4471 g) was added drop wise by an addition funnel. The mixture was left to stir for 2 h at low temperature. An orange precipitate was collect by vacuum filtration, dried, and then used without any further purification to avoid decomposition.

5-(bis(3-phenylpropyl)amino)-2-nitrosophenol. Compound 80c was dissolved in 15 mL of EtOH. HCl (8 ml, 32%) was added to the solution. The solution was kept on an ice bath, stirring for the remainder of the reaction to maintain a temperature at 0-5 °C. Over a period of 1 h, an 8 mL of solution of sodium nitrite (0.4471 g) was added drop wise by an addition funnel. The mixture was left to stir for 2 h at low temperature. There was not any precipitate due to the organic solvent added for solubility issues. The reaction mixture was decanted into another round bottom flask and was put on a rotovap. All solvent was removed under reduced pressure at approximately 60 °C. This left a yellow-orange residue tinged with brown impurities. DCM was added to the mixture to dissolved the impurities. The flask was placed into a sonicator where the impurities went into solution and the precipitate formed a globular pellet in the center. The pellet was scooped out and washed with 5 mL of DCM. No further purification was carried out due to cited instability of the nitroso compound; 46% yield

5-(butylamino)-4-methyl-2-nitrosophenol. Compound 81e (1.87 g, 10.42 mmol) was dissolved in 25 mL of EtOH. The solution was cooled to 0 °C on an ice bath. Sodium nitrite (800
mg, 11.59 mmol) was dissolved in 6 mL of D.I. water and was added to the reaction mixture drop wise over the course of an hour. The reaction mixture was left to stir for an addition 2 h on ice. The product was concentrated under reduced pressure to remove all traces of water. It was then dissolved in the minimum amount of ethanol and filtered to remove salts. The product was concentrated again and was used in the next step without further purification. Product was obtained with 94% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 0.90 (t, 3H, $J = 8$Hz), 1.34 (sex, 2H, $J = 8.0$Hz), 1.62 (quin, 2H, $J = 8.0$Hz), 2.20 (s, 3H), 3.53 (t, 2H, $J = 8$Hz), 6.58 (s, 1H), 7.36 (s, 1H), 10.47 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 13.07, 16.97, 19.06, 29.64, 43.94, 95.26, 120.05, 130.71, 144.29, 162.77, 166.70; m.p. 156-158 °C.

**4-methyl-2-nitroso-5-((3-phenylpropyl)amino)phenol.** Compound 81f (659 mg, 2.73 mmol) was dissolved in 25 mL of EtOH. The solution was cooled to 0 °C on an ice bath. Sodium nitrite (207 mg, 3 mmol) was dissolved in 2 mL of D.I. water and was added to the reaction mixture drop wise over the course of an hour. The reaction mixture was left to stir for an addition 2 h on ice. The product was concentrated under reduced pressure to remove all traces of water. It was then dissolved in the minimum amount of ethanol and filtered to remove salts. The product was concentrated again and was used in the next step without further purification. MS (ESI): 270.2 (M$^+$); $\lambda_{max}$= 355 nm (MeOH).

**9-(diethylamino)-2-hydroxy-5H-benzo[a]phenoxazin-5-one.** Compound 82a (0.771 g, 3.97 mmol) was mixed with 1,6-dihydroxynaphthalene in DMF and then refluxed at 70 °C for 2 h to afford the product. The reaction mixture was dried by rotary evaporation and the product was crystallized from MeOH/ether. Product was then purified by normal phase chromatography eluting with 1% MeOH in DCM. 60% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.49 (s, 1H), 7.96 (d, 1H, $J = 8.0$ Hz), 7.87 (s, 1H), 7.54 (d, 1H, $J = 12.0$ Hz), 7.09 (d, 1H, $J = 8.0$ Hz), 6.78
(d, 1H, J = 8.0), 6.13 s, 1H), 1.15 (t, 6H, J = 8.0 Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 181.12, 160.04, 151.07, 150.15, 145.85, 138.02, 133.20, 130.28, 126.94, 123.31, 117.80, 109.41, 107.55, 103.48, 95.42, 43.89, 11.89; MS (ESI): 336 (M$^+$); $\lambda_{max}$=548 nm (MeOH); m.p. >230 °C

**9-(dibutylamino)-2-hydroxy-5$H$-benzo[a]phenoxazin-5-one.** Compound 82b was mixed with 1,6-dihydroxynaphthalene in DMF and then refluxed at 70 °C for 2 h to afford the product. Solvent was removed under reduced pressure and the residue was purified via regular phase column chromatography eluting with 1% MeOH in DCM. The product was still slightly impure. Washing the product with 1 mL of EtOAc and then decanting off the liquid left a green solid once dry. 52% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.26 (d, 1H, J = 8Hz), 7.59 (d, 1H, J = 8Hz), 7.15 (d, 1H, J = 8Hz), 6.66 (d, 1H, J = 8Hz), 6.46 (s, 1H), 6.34 (s, 1H), 6.34 (s, 1H), 5.66 (m, 1H), 3.41 (t, 4H, J = 8Hz), 1.66 (t, 4H, J = 8Hz), 1.44 (q, 4H, J = 8Hz), 1.03 (t, 6H, J = 8Hz); MS (ESI): 390 (M$^+$); m.p. 224-226 °C

**9-(butylamino)-2-hydroxy-10-methyl-5$H$-benzo[a]phenoxazin-5-one.** 41% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 0.94 (t, 3H, J = 8Hz), 1.40 (sex, 2H, J = 8Hz), 1.61 (quin, 2H, J = 8Hz), 2.21 (s, 3H), 3.30 (t, 2H, J = 8Hz), 6.36 (s, 1H), 6.63 (s, 1H), 7.135 (dd, 1H), 7.47 (s, 1H), 7.90 (d, 1H, J = 8Hz), 8.00 (d, 1H, J = 8Hz)

**2-hydroxy-10-methyl-9-((3-phenylpropyl)amino)-5$H$-benzo[a]phenoxazin-5-one.** 28% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.94 (quin, 2H, J = 8Hz), 2.20 (s, 3H), 2.71 (t, 2H, J = 8Hz), 3.32 (t, 2H, J = 8Hz), 6.32 (s, 1H), 6.59 (s, 1H), 7.135 (dd, 1H), 7.18-7.33 (m, 5H), 7.47 (s, 1H), 7.92 (d, 1H, J = 4Hz), 8.01 (d, 1H, J = 8Hz);

**3-(ethylamino)phenol.** 3-aminophenol (4.00 g, 36.7 mmol), potassium carbonate (5.07 g, 36.7 mmol), and ethyl iodide (2.95 mL, 36.7 mmol) were combined in a flask with 20 mL of DMF. The reaction mixture was stirred and heated for 2 h at 100 °C. It was then concentrated
under reduced pressure to remove all DMF. The residual oil was loaded onto a regular phase column and eluted with a 1:4 EtOAc/Hexanes mobile phase which was removed under reduced pressure. The product was a light brown oil and was recovered with a 51% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.13 (t, 3H, $J = 8$Hz), 2.96 (q, 2H, $J = 8$Hz), 5.99 (m, 3H), 6.83 (t, 1H, $J = 8$Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 14.36, 37.35, 98.89, 103.09, 103.59, 129.37, 150.18, 158.10.  

**9-(diethylamino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 60% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.814 (s, 1H), 9.752 (s, 1H), 8.748, (d, 1H, $J = 8$ Hz), 8.425 (d, 1H, $J = 8$ Hz), 7.968 (t, 1H, $J = 8$ Hz), 7.858 (t, 1H, $J = 8$ Hz), 7.799 (d, 1H, $J = 10$ Hz), 7.230 (d, 1H, $J = 10$ Hz), 6.960 (s, 1H), 6.797 (s, 1H), 3.655 (q, 4H, $J = 7.2$ Hz), 1.236 (t, 6H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 161.19, 153.93, 151.35, 148.07, 133.69, 132.91, 132.85, 131.66, 129.97, 129.57, 124.47, 124.32, 122.89, 115.44, 96.91, 96.21, 45.84, 13.07; $\lambda_{\text{max}}$ = 626 nm (MeOH); m.p. 285-287 °C

**9-(dibutylamino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 34% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.776 (s, 2H), 8.688 (d, 1H, $J = 8$ Hz), 8.385 (d, 1H, $J = 8$ Hz), 7.926 (t, 1H, $J = 7.6$ Hz), 7.817 (t, 1H, $J = 7.6$ Hz), 7.27 (d, 1H, $J = 9.2$), 7.151(d, 1H, $J = 8.8$ Hz), 6.862 (s, 1H), 6.743 (s, 1H), 3.540 (t, 4H, $J = 7.2$ Hz), 1.584 (quin, 4H, $J = 7.2$ Hz), 1.391 (sex, 4H, $J = 7.2$ Hz), 0.954 (t, 6H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 161.30, 154.26, 151.44, 148.06, 133.97, 132.86, 131.76, 130.01, 129.55, 124.54, 124.40, 123.07, 115.49, 97.06, 96.39, 51.38, 29.65, 19.96, 14.27;

**9-(bis(3-phenylpropyl)amino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 29% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.906 (s, 1H), 9.832 (s, 1H), 8.762 (d, 1H, $J = 8.0$ Hz), 8.449 (d, 1H, $J = 8.4$ Hz), 7.974 (t, 1H, $J = 8.0$ Hz), 7.866 (t, 1H, $J = 7.6$ Hz), 7.772 (d, 1H, $J =
9.2 Hz), 7.346-7.213 (m, 10H), 7.151 (d, 1H, J = 8.0 Hz), 6.824 (s, 1H), 6.812 (s, 1H), 3.619-3.584 (m, 4H), 2.717-2.679 (m, 4H), 1.945-1.912 (m, 4H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 161.47, 154.18, 151.60, 148.00, 141.71, 134.27, 133.00, 132.77, 131.79, 130.15, 129.52, 128.89, 128.75, 126.46, 124.60, 124.48, 123.06, 115.40, 97.07, 96.46, 51.07, 32.57, 29.19; $\lambda_{\text{max}}$ = 628 nm (MeOH); m.p. 218-220 °C

**9-(ethylamino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 20% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.596 (d, 1H, J = 8.0 Hz), 8.316, (d, 1H, J = 8.0 Hz), 7.876 (t, 1H, J = 7.2 Hz), 7.770 (t, 1H, J = 7.2 Hz), 7.623 (d, 1H, J = 8.8 Hz), 6.965 (d, 1H, J = 8.8 Hz), 6.63 (s, 1H), 6.652 (s, 1H), 3.311-3.294 (m, 2H), 1.250 (t, 3H, J = 7.2 Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 160.82, 156.37, 151.08, 133.00, 132.66, 131.58, 130.10, 129.79, 124.36, 124.18, 122.82, 96.66, 38.29, 14.19; $\lambda_{\text{max}}$ = 610 nm (MeOH); m.p. 198-200 °C

**9-(butylamino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 12% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.641 (s, 2H), 8.661 (d, 1H, J = Hz), 8.468 (s, 1H), 8.368 (d, 1H, J = Hz), 7.909 (t, 1H, J = Hz), 7.798 (t, 1H, J = Hz), 7.678 (d, 1H, J = Hz), 7.031 (d, 1H, J = Hz), 6.736 (s, 2H), 3.291 (m, 2H), 1.607 (m, 2H), 1.420 (m, 2H), 0.959 (m, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 160.85, 156.65, 151.22, 133.06, 132.69, 131.70, 130.23, 129.82, 124.43, 124.25, 122.90, 96.70, 43.23, 30.74, 20.14, 14.13;

**9-((3-phenylpropyl)amino)-5H-benzo[a]phenoxazin-5-iminium perchlorate.** 12% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.714 (s, 2H), 8.788 (d, 1H, J = Hz), 8.552 (s, 1H), 8.454 (d, 1H, J = Hz), 7.982 (t, 1H, J = Hz), 7.872 (t, 1H, J = Hz), 7.798 (d, 1H, J = Hz), 7.321-7.217 (m, 5H), 7.106 (d, 1H, J = Hz), 6.832 (s, 2H), 2.736 (m, 2H), 1.950 (m, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 161.10, 156.63, 151.55, 141.78, 133.51, 132.89, 131.87, 130.30,
130.02, 128.86, 128.79, 126.42, 124.55, 124.42, 123.06, 96.83, 43.01, 32.87, 30.44; \lambda_{\max} = 616 \text{ nm (MeOH)}
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APPENDICES

Appendix A — $^1$H NMR, $^{13}$C NMR, HRMS, and IR Spectra

All $^1$H NMR and $^{13}$C NMR spectra were obtained using a 400 MHz Bruker Avance spectrometer and were processed using the Topspin program. All NMR spectra were post processed for this thesis using Mestrenova Lite. High resolution mass spectra were taken using either a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer provided at the Georgia State University Mass Spectrometry Facility or from a Waters Micromass LCT TOF ES+ Premier Mass Spectrometer.
H₂N

OH

NMR Solvent: DMSO-d₆
NMR Solvent: DMSO-\(d_6\)
in DMSO-\textit{d}_6
in DMSO-$d_6$
NMR Solvent: CDCl₃
NMR Solvent: CDCl$_3$
NMR Solvent: CDCl$_3$
minor impurities: acetone and ethyl acetone
NMR Solvent: CDCl₃
NMR Solvent: CDCl₃
NMR Solvent: CDCl₃
NMR Solvent: DMSO-$d_6$
NMR Solvent: DMSO-$d_6$
NMR Solvent: CDCl₃
NMR Solvent: ACN-$d_3$
NMR Solvent: ACN-$d_3$
**Figure 1:** TOF MS ES+

1: Nile Red for paper 453 (8.914) Cm (446:459)

**1: TOF MS ES+**

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**Figure 2:** IR Spectra

2968.73 cm⁻¹, 76.34% T

**Figure 3:** VM-3

Exact Mass: 336.15
VM-25
NMR Solvent: DMSO-$d_6$
VM-25
NMR Solvent: DMSO-\textit{d}_6
VM-23
NMR Solvent: DMSO-d₆
VM-47
NMR Solvent: DMSO-<i>d</i><sub>6</sub>
VM-47
NMR Solvent: DMSO-$d_6$
VM. 47, ESIPOS, HENARY. 05/20/2015 #220-229  RT: 3.09-3.21  AV: 10  NL: 1.58E9
T: FTMS + p ESI Full ms [100.00-1000.00]

Relative Abundance

Exact Mass: 318.16
NMR Solvent: DMSO-$d_6$ + drop of $D_2$O
NMR Solvent: DMSO-$d_6$
VM_MeOH5_ESIPOS_HENARY_05202015 #217-231 RT: 3.05-3.24 AV: 15 NL: 8.21E7
T: FTMS + p ESI Full ms [100.00-1000.00]

Exact Mass: 374.22

374.2223
z=1
318.1597
z=1
290.1285
z=1
380.1748
z=1

VM-38

m/z

Relative Abundance

374.2223
z=1
318.1597
z=1
290.1285
z=1
380.1748
z=1
VM-46
NMR Solvent: DMSO-$d_6$
VM 46 ESIPOS HENARY 05202015 #213-223  RT: 3.00-3.14  AV: 11  NL: 4.00E8
T: FTMS + p ESI Full ms [100.00-1000.00]

Exact Mass: 498.25

498.2530
z=1

266.1171
z=1
VM-41
NMR Solvent: DMSO-$d_6$
VM-41

NMR Solvent: DMSO-$d_6$ + drop of $D_2$O
VM-44
NMR Solvent: DMSO-$d_6$
VM_44_ESIPOS_HENARY_05202015 #178-184 RT: 2.49-2.57 AV: 7 NL: 1.68E9
T: FTMS + p ESI Full ms [100.00-1000.00]

318.1596
z=1

Exact Mass: 318.16
VM-45

NMR Solvent: DMSO-$d_6$
VM_45_ESIPOS_HENARY_05202015 #216-225  RT: 3.03-3.16  AV: 10  NL: 1.01E9
T: FTMS + p ESI/Full ms [100.00-1000.00]

Exact Mass: 380.18