Synthesis and Photophysical Properties of Phenoxazine Analogs Designed as Potential Antimalarials

Carl Mufungwa Kananda
ckananda1@student.gsu.edu

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SYNTHESIS AND PHOTOPHYSICAL PROPERTIES OF PHENOXAZINE ANALOGS DESIGNED AS POTENTIAL ANTIMALARIALS

by

CARL MUFUNGWA KANANDA

Under the Direction of Maged M. Henary, Ph.D.

ABSTRACT

The phenoxazine core has been explored in the development of chromophoric systems that have seen use as fluorescent probes and therapeutic agents in combating illnesses of many forms. This thesis reports the synthesis and evaluation of some novel phenoxazine and benzo/a/phenoxazine analogs. Phenoxazinium are charged lipophilic molecules and the recent resurgence of π-Delocalized Lipophilic Cations (DLC) hypothesis in the combat against protozoan illnesses has prompted focus on the synthetic exploration of the derivatization of their architectures in medicinal chemistry. The principle aim of these modifications were to synthesize a focused library of systematically modified analogs catering to the enhancement of related “drug like” properties found throughout the literature of their development as antimalarials.

INDEX WORDS: Phenoxazine, Benzophenoxazine Dyes, Nile blue, Delocalized Lipophilic Cations (DLC), Malaria
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ANALOGS DESIGNED AS POTENTIAL ANTIMALARIALS

by

CARL MUFUNGWA KANANDA

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science
in the College of Arts and Sciences
Georgia State University
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by

CARL MUFUNGWA KANANDA

Committee Chair: Maged Henary

Committee: Maged Henary
Donald Hamelberg
Jun Yin

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2018
DEDICATION

In memory of Tyler Crawford.
ACKNOWLEDGEMENTS

I would like to thank my P.I. Dr. Maged Henary for the immense opportunity and support during the completion of this project. I would like to acknowledge members of my committee, Dr. Donald Hamelberg and Dr. Jun Yin. I am indebted to past and present members of my research group who have assisted and have offered a close camaraderie during the completion of my research. A special thanks to Vincent Martinez for assisting me with the initial design of the project, Mathew Laramie for always sharing valuable insights, Corey Holder for the revision of my thesis, and Eddie Soriano, Peace Ineza, Fahad Marmarchi, Laura Rotolo, and Abdelbasset Farahat, Nicholas Mulkey. I want to thank Dr. David Boykin for always keeping his door open and the invaluable help in establishing the collaboration that made this special project possible, and Dr. Sergio Wittlin and his colleagues at TPH for their collaboration. I would like to thank my parents, siblings, and all my family and friends for their support. Lastly, I would like to thank my best friend, Ariana Jones, thank you for always believing in me and being by my side.
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1 INTRODUCTION

As cited in the unofficial excerpts of the modernization of man, many years ago a hunter went out for a hunt after a lightning storm. The hunt was unsuccessful because most of the vegetation had been destroyed by the fire from the skies. Frustrated, he began deliberately swinging his amber glowing spear at a nearby brush. Soon after, the bush erupted in flames and from it emerged birds with sparked feathers. The birds died shortly after. The hunter attracted by the aroma of freshly combusted meat, hastily gave it a tasting try and to his pleasant surprise the bird was delicious. He coined the phenomena Baba Ku, in honor of his father Kuruk. Excited to share his recent discovery with the rest of his village, he swam back inshore to share the findings with his village chief, who after hearing the story and tasting the bird, ordered every man in the village to swing their spears at every bush on the island until the fiery birds ascended. After days of tirelessly beating the heck out of every bird perched bush, most of the fruitful vegetation in the village was destroyed. All the villagers were exhausted with no reliable sighting of the bird. With their main source of food gone, the villagers were angry and starving. Alas for the hunter and his family, they were banished from the village that night. They found shelter in a cave excavated by the waves on the shorelines, and once again the hunter made another discovery. He found that striking the rock bearing end of his hunting spear against the rigid walls of the cave, again out of frustration, created sparks which were reflected by the nearby waves, illuminating the entire cave and surroundings from where he was able to foresee an indigenous populace invade the hunger weakened village. In a matter of months all of the village’s resources including its people, were harnessed and plundered by the invaders. All of it, except for the hunter and his family, was destroyed.

In an analogous development, the middle age saw most of Europe set sail in treacherous waters beyond the horizon of the world they knew. Some in the name of monarchs, some in the name of
God, some in the name of science—but most with dreams of discovering and conquering a new world. When European ships washed on the new shores, they hastily harnessed and carried back tales of distant indigenous civilizations and the plunders of their lands—many of which were unknown to Europe. Part of the plunder included plants known by the natives for their remedial powers in the treatment of many maladies. The wild untamed flora the native co-existed with served as their apothecary, providing them with many useful shrubs and trees such as the Eysenhardtia polystachya (commonly known as kidneywood tree) used by the native Aztec as a diuretic. During the early days of European colonies in the New World, many settlers and explorer succumbed in great numbers to diseases. One particularly, inflicted through a mosquito bite and later discovered to be the result of parasitic infection, wreaked havoc across Europe and in the New World. The cinchona tree was a native curative plant used in the treatment of the potent disease, malaria. The kidney wood and cinchona tree played a revolutionary role in the development of remedies; however, centuries later, observations made in the experimental investigation of their curative abilities led to many ground-breaking advances in science. In the 15th century, a Spanish physician and botanist, “Don” Nicolas Monardes noticed a “peculiar blue color” illuminating from the water infusion of the kidney wood tree. This same illuminating phenomenon was observed by the English scientist Sir John Frederick William when holding a solution of quinine, the compound isolated from the barks of the cinchona tree used in treating malaria, in sunlight. The illumination was the result of a phenomenon that captivated the attention of many scientists, particularly an Irish physicist and mathematician, Sir George Gabriel Stokes, who later extensively studied and described the process behind the phenomenon.

The following accounts, despite one holding greater truth than the other, share the important notion of serendipitous discovery associated with illumination. The ability to shed light on things
has been one of the biggest influencers behind the advancement of humans. From the use of wood fire by prehistoric people to the incandescent light bulb in recent times, illumination has seen its paramount use as a method of revealing secrets of the dark. In the story of the hunter and his family, the accidental discovery of manufactured illumination allowed them to foresee and escape invading savages. In modern day biology and biomedical sciences, the kind of illumination described by European scientists in the 15th century has played a vital role in assisting scientists today detect threats and treat humans from invasions by pathological hosts of many forms.

1.1 Absorbance and Fluorescence of chromophores

The systems responsible for the coloration of objects are known in chemistry as chromophores. The color of objects is directly related to the ability of chromophores to absorb light. Simply put, the color seen corresponds to the complement of visible light wavelengths not absorbed by chromophores. Even more interesting is that the chemical basis of sight, itself relies on the ability of a chromophore, retinal, to absorb light. Other examples of very useful biological chromophores in living systems include heme, the active site of the oxygen carrying protein hemoglobin, and melanin, the molecules responsible for the coloration of the skin and absorption of the sun’s ultraviolet (UV) radiation (Figure 1.1).
The ability of most organic chromophores to absorb light lies in their conjugation— the successive occurrence of single and multiple bonds. Inspection at the atomic level of these systems has enlightened scientists on the quantum phenomena associated with their structure and their relationship between their color and light. In conjugated systems, valence electron mobility is not restricted to the respective orbital of their unsaturated π connection. Instead, the electrons are omnipresent, freely roaming throughout the conjugated system, occupying a connected network of overlapped electron clouds called a delocalized π-orbital. When this is the case, an energy difference can be observed in the molecule where the electrons in the highest occupied molecular orbital or HOMO occupy a lower more energetically favorable position. Electrons present in this state can be stimulated by light corresponding to the difference in the energy gaps between the lowest unoccupied molecular orbital (LUMO) and the HOMO orbital to a higher corresponding energy level or an excited state. This process is simply referred to as absorption, analogous to the intake of a photon. In general, the length in the conjugation of a chromophore is directly related to
the wavelength of the light absorbed i.e. the longer the delocalization of π electron, the lower the HOMO to LUMO energetically difference, thus the larger the wavelength value of absorption ($\lambda_{\text{max}}$) on the electromagnetic spectrum.8

Another feature reserved to conjugated systems, is their ability to spontaneously release the absorbed light. The phenomena of the absorption and subsequent release of light by molecules have been extensively studied and explained in literature through the use of the Jablonski diagram shown in Figure 1.2. Electrons that have been excited from their ground state, can expeditiously relax through non-radiative decay to the lowest vibrational excited state or their first singlet excited electronic state ($S_1$). This is known as internal conversion. The continuous relaxation of electrons back to the initial ground state solely through vibrational states result in the generation of heat. Photoluminescence occurs when electrons return to their ground states, simultaneously emitting the absorbed light. There exist differences in the physical manifestations (photoluminescence) of the emitted energy which is governed by the pathway the electron utilizes to return to its ground state. A distinction of the pathway is the spin state of the electrons, the parameter of intrinsic angular momentum, afore relaxation. Electrons with varying spins states utilize different relaxation pathways. When an electron returns to the ground state from its excited singlet state ($S_1$) through the spontaneous emission of a photon, fluorescence occurs. A fluorophore or a fluorochrome, similar to a chromophore, is a molecule that can re-emit light upon light excitation. Seldom, an electron can enter an alternative forbidden spin state known as a triplet state ($T_1$) through intersystem crossing. Relaxation of electrons from the triplet state to the ground state happens at a much slower rate than fluorescence and is known as phosphorescence.9
1.2 Dyes

While a chromophore only directs to the part of the molecule responsible for the absorption of light, there are other structural features of light absorbing systems that also play roles in assisting coloration. An entire molecule comprised of a chromophore appended with functional groups (auxochromes) to enhance its coloring abilities is simply known as a dye. Pigments are larger molecules of analogous framework typically defined by their insolubility in most mediums. While there exist many natural dyes, most from animal and plant extracts, the majority of dyes are man-made.

As demands for drugs to combat malaria grew because the virulence of the many parasites responsible for the illness was increasing, organic chemists ran to their laboratory benches to attempt to synthesize analogs of the only effective cure for the disease, quinine. While attempting
to prepare synthetic quinine, Sir William Henry Perkin, a British scientist, discovered that rinsing the dark gunk of the reaction with alcohol created a beautiful purple mess. Mauveine was the name given to this concoction which consisted of several forms of a tricyclic aromatic nitrogen cation (Figure 1.3). It was the first account for the synthesis of an organic dye, marking what is considered today, the cornerstone for the synthetic dye industry and industrial synthetic chemistry as a whole.

![Figure 1.3 Structures of major analogs of Perkin's Mauveine dyes](image)

Organic dyes and their chemistries have since evolved from simple fused aromatic compounds used mainly as colorant in the food and textile industries, into highly modifiable scaffolds used in a wide range of industrial applications and cutting edge technologies. Many of the popularly used scaffolds include carbocyanines, BODIPY, rhodamine, coumarin, squaraine, oxazine and azo-based dyes (Figure 1.4). Dyes have found uses as fluorescent probes for biomolecular labeling and imaging technology. Fluorophores can be specially designed to respond to specific stimuli to study the morphology in living systems. They can be appended to protein to understand folding mechanism and structures, or to a ligand to explore binding to a receptor and to understand the target and mode of action of therapeutic treatments. They can also be rationally designed to have structure inherent targeting potential. Their ability to act as light transistors has seen their increased use in industrial settings as photosensitizers in solar panels.
Figure 1.4 Scaffolds of popular organic dyes

1.3 Phenoxazine and benzo(a)/Phenoxazine

1.3.1 Introduction

Oxazine refers to any members of a class of molecules containing all four of earth’s most biochemically relevant elements—carbon, oxygen, nitrogen, and hydrogen bound together in such a way that carbon (C) and nitrogen (N) are in a six membered heterocyclic ring containing three sp² hybridized carbons. Illustrated in Figure 1.5 are the three main classes of oxazine, named 1,2-, 1,3-, 1,4-oxazine in reference to the positioning of the oxygen and nitrogen atom on the ring. The extension of 1,4-oxazine with two fused phenyl rings on either adjacent faces, hence named phenoxazine, represents an important system in chemistry known for its stability, planarity, and lipophilic nature. When phenoxazine is flanked by two nitrogen functionalities, a charged imine and a neutral amine, the scaffold takes on the suffix “ium”.

The two groups act as acceptor and
donor of π-electron across the conjugated system accounting for desirable photophysical characteristics of the molecule. Consequently, phenoxazinium analogs have found use in various fields as fluorescent probes.\(^27\) Benzo/\(a\)/phenoxazinium are analogs of the phenoxazine family bearing a fused phenyl at the “\(a\)” face of one their adjacent benzene rings. Nile blue is a renowned cationic dye belonging to the benzo/\(a\)/phenoxazine family frequently used as a histological stain.\(^{28-30}\)

![Diagram of oxazine derivatives]

**Figure 1.5** General nomenclature of oxazine, and analogous derivatives and dyes

### 1.3.2 Synthesis

The preparation of phenoxazine and benzophenoxazine dyes have not much varied since the original literature mention of the rugged cyclization of catechol with \(o\)-aminophenol, only the amine have been replaced by much more reactive nitroso group.\(^31\) The modernized synthesis of phenoxazine and benzophenoxazine dyes usually begins with the functionalization of 3-
aminophenol at the \( N \) position. The primary amine at the 3-position is a good nucleophile that can be functionalized with various substituents through a substitution reaction to acquire primary or secondary amines. Another popular method to include varied amino substituents onto the phenol is through the reaction of 3-halophenol with the desired amino functionality using C-N coupling methods such as the Buchwald-Hartwig Cross Coupling Reaction.\(^{32}\)

![Chemical diagram]

**Figure 1.6 General synthesis of phenoxazine and benzo\( a \)/phenoxazine analogs**

The next steps in the formation of the dye calls for the conversion of the aminophenol to 5-amino-2-nitrosophenol followed by its reaction with 3-aminophenol or 1-aminonaphthalene in acetic acid to yield the phenoxazinium and benzophenoxazinium dye, respectively (Figure 1.6). Unfortunately, the yields for these reactions remain low due to the thermal sensitivity of the nitroso specie and the promptness of the reaction to cyclize into many other undesired products in the last step. Inclusion of varied substituents on either terminal nitrogen has called for the use of ethanol as solvent and catalytic amounts of some mineral acids such as hydrochloric acid or perchloric acid as reaction promoters, to replace the use of acetic acid in attempts to improve the efficiency of this
reaction. This method has afforded simple substituents on the terminal nitrogen at improved yields compared to the conventional methods, but when more “exotic” groups are desired on the dye, more robust reaction conditions are required such as the use of elevated reaction temperature using high boiling solvent, longer reaction times, and expensive/toxic catalysts. These conditions are unfavorable and can lead to the decomposition of the thermally sensitive nitroso intermediate whilst also promoting the formation of unwanted side product, resulting in challenging work ups and purification procedures. These challenges often result in the loss of product yield to an already yield greedy reaction.

It is also important to note that the electronics and steric nuisance of some complex aminophenols, such as those containing cyclic or aromatic amines, or N-substituted functionalities with strong electron donating/ withdrawing capability, do not often allow for their conversion to the corresponding nitroso form necessary for formation of the dye. To circumvent this issue, activating the ortho position of the aminophenol using alternative nitrogen containing groups have been devised by organic chemist.

**1.3.2.1 Diaryl azo route**

A method using a diarylazo intermediate has been employed to synthesize phenoxazine and benzophenoxazine analogs\(^3\). This protocol has proved successful in incorporating “privileged” functionalities on both N-terminals. It begins with the diazotization of \( p \)-nitroanaline into an arenediazonium salt. Diazonium species are notorious in organic synthesis for their reactivity. They can react with nucleophiles such as halides, hydride, cyanide, and hydroxide among many others, where the benzene can favorably lose the diazene group as nitrogen gas to yield their corresponding substituted benzenes. They are also known to readily react with
ortho/para-activated benzenes through a diazonium coupling reaction to form a para azo-coupled product.

In the case of the synthesis of oxazine dyes, a meta-aminophenol is reacted with a diazonium salt to form a diarylazo molecule. Reaction of the azo compound with 3-aminophenol or 1-aminonaphthalene in the presence of perchloric acid yields an oxazine dye (Figure 1.7.). Advantages of this protocol includes simpler work up and purification methods which translate to reduction of loss of yield. It is important to note when highlighting the diversity of this protocol for the synthesis of oxazine dyes, that either aromatic reactant can be converted into the azo form and reacted with the corresponding aromatic lacking the azo group to form the oxazine dye as shown in Figure 1.7.
1.3.2.2 Diaryl ether route

Another recently developed route extended from the diarylazo intermediate has led to the synthesis of even more structurally diversified oxazine analogs than any previously reported methods with improved yields. In the previously reported azo method, the diaryl azo intermediate could easily be obtained through the azo coupling. Synthetic challenges arose in the final condensation step of the azo intermediate with the corresponding non-azo aminophenol. In order to address the short comings of this protocol, Cornish et al. hypothesized that concerting the molecules by bridging the two reactants together before forming the azo intermediate could potentially promote the desired cyclization into the cationic compounds. This was accomplished
experimentally through the initial paring of 3-aminophenol with aryl iodide in a copper(I)-promoted reaction to form a tethered diaryl ether intermediate. The reaction of the intermediate with 4-nitrobenzene diazonium tetrafluoroborate resulted in the formation of the diazo compound, which upon heating with p-toluenesulfonic acid in ethanol cyclized into the desired dye (Figure 1.8).34

![Figure 1.8 Diaryl ether route](image)

1.4 Biological application of oxazine dyes

Phenoxazine and benzophenoxazine analogs have been used in an array of biological applications. Historically, their desirable stability and photophysical properties have made them particularly attractive for photonic applications such as fluorescence imaging, fiber optics, and in phototherapies among many others. Oxazine dyes are also charged, planar, lipophilic molecules. Such features are key in increasing their interaction with lipophilic macromolecules, thus increasing their therapeutic potential. Recent work in the field of organic chemistry has led to the emergence of new and efficient methods for the design and preparation of phenoxazine and benzophenoxazine analogs. These refined synthetic tools have unlocked the construction of structurally diverse analogs resulting in a resurgence of their application in cutting edge
technologies. This section will cover recent advances in literature in the biological application of oxazine based structural analogs. It will be divided in two parts. The first part will highlight the applications of phenoxazinium dyes as fluorescent probes in the past 5 years with a short mention of benzophenoxazinium dyes as their applications have been thoroughly covered in a review published by our group. The second part will be a compressed review of their application as small molecule therapeutic agents, focusing on their use as delocalized lipophilic cations.

1.4.1 Fluorescence application

1.4.1.1 Phenoxazine

Small molecule organic fluorescent probes are powerful tools in the elucidation of biological processes. Suitable probes for biological application must follow certain photophysical requirements for in vivo optical application such as minimal photoinduced damage to biological samples, and have significant bathochromic shift for relatively high signal-to-background ratios. Certain physicochemical properties such as size, lipophilicity, solubility, and ionization state must also be met in accordance to the Lipinski and Veber rules. Phenoxazinium and benzophenoxazinium are a class of oxazine fluorescent compounds that have found use in fluorescence biological application due to their many desirable properties. Modification to their architectures to enhance their usage and address drawbacks as fluorescent probes have been addressed in recent literature.

Certain functional units are employed in medicinal chemistry as structural modulators of the photophysical properties of a chromophore, while other are suited to address the physicochemical profile of the molecules. In the design of a fluorescent probe with enhanced properties for biological application, Hell et al. addressed the two in synergy using a common structural motif. Analogs of ATTO655, an oxazine probe used in single-molecule localization
microscopy, were synthesized featuring a primary phosphate group 6 to enhance both its fluorescence quantum yields and solubility for the improved imaging of immunolabeled cytoskeletal protein vimentin in mammalian cells (Figure 1.9).37

\[ \text{Figure 1.9 Synthesis of phosphorylated ATT655 analog} \]

Some probes are designed to feature a mechanism of fluorescence ignition as a response to a stimulus. When the mode of ignition is light induced, it is called photoactivation. Photoactivatable fluorophores (PAFs) are important in fluorescence application. In in vivo cell imaging, the photoactivation of a fluorophore can permit for real time monitoring of dynamic cellular processes.38 Cornish et al. adapted the phenoxazine scaffolds in designing a photoactivable probe PA-Oxazine for live cell imaging.39 In their work, they incorporated an azido group at receptor amine position and an acyl moiety onto the central nitrogen ring that could be cleaved by UV-light, leaving behind the fluorescent compound 10, shown in Figure 1.10. The probe was used in the protein specific labeling of living mammalian cells through linkage to the protein specific trimethoprim (TMP) tag.
Figure 1.10 Synthesis of azido-acyl caged photoactivable oxazine analogs

Phenoxazinium probes can also be designed to respond to changes in pH levels. Ge et al. developed a series of pH sensitive phenoxazinium analogs by appending substituents to their donor amines that could serve as protonation sites in developing lysosome targetable probes\textsuperscript{40-41} (Figure 1.11). Their receptor amines were readily protonated/deprotonated or appended with pH sensitive moieties that could undergo protonation upon change in pH level, which elicited a change in the emission profile of the dyes.

Figure 1.11 Protonation/deprotonation mechanism of phenoxazinium pH based probes.
Pickup et al. used click chemistry to append **651-Blue Oxazine** dye to glucose/galactose-binding protein (GBP) to develop a probe 11 for the fluorescence sensing of glucose (Figure 1.12). Phenoxazinium dyes are solvatochromic systems that have been shown to experience bathochromic shift in polar environments. Upon binding of the GBP-Blue Oxazine 11 to the protein, there is conformational change which increase the polarity in the binding site of the enzyme resulting in a noticeable increase in fluorescence intensity of the fluorophore.

![Figure 1.12](image.png)

**Figure 1.12** Copper mediated click reaction of Blue Oxazine azide to GBP-alkyne to synthesize a GBP-Blue Oxazine glucose sensing probe.

Another common application of phenoxazinium based fluorescence is in developing probes for metal sensing. Metal sensors can be obtained by appending known chelating ligand onto the amine of the phenoxazine. Lu et al. investigated the use of di(2-picolyl)amino moiety as a metal binding domain on the oxazine scaffold to develop suitable probes for the intracellular sensing of divalent metal ions. Appending di(2-picolyl)amino directly onto one of the terminal nitrogen showed the selectivity of probe 12 towards copper(II) ions. In another study, they demonstrated the versatility of the oxazine scaffold in metal sensing chemistry. Increasing the length of the connecting chain of the binding domain to the fluorophore by two carbons resulted in a probe 13
more selective towards the sensing of the larger zinc(II) ion perhaps due to the increase range of motion of the binding ligand (Figure 1.13). \(^{45}\)

![Figure 1.13 Di(2-picoly)amino based phenoxazinium probes for divalent metal sensing.](image)

Photoinduced electron transfer (PET) quenching is a popular mode of fluorescence turn on where a donor molecule’s ability to transfer an excited state electron to a neighboring receptor chromophore is hindered, resulting in the increase in fluorescence intensity of the acceptor fluorophore. \(^{46}\) Kobertz \textit{et al.} developed a NIR probe 14 constituting of the known triazacryptand (TAC) binding moiety (donor) covalently linked to an oxazine fluorophore (receptor) for detecting changes in intracellular and extracellular potassium ion (K\(^+\)) concentrations using a PET quenching sensing mechanism. \(^{47}\) Figure 1.14 depicts the mechanism of fluorescence quenching; when the metal is lodged in the binding domain of the probe PET to the chromophore is quenched, resulting in enhanced fluorescence emission of the fluorophore.
Dyes can be built into nanoparticles to enhance their use as fluorophores. Nanoparticles can act as surfaces for the adhesion of small molecules chromophores in the design of fluorescent probes for in vivo use. The nanoparticle must be judiciously selected to have specific features that allows for their appendage without sacrificing the optical performance of the fluorophore, while improving physicochemical properties of the probe such as specific cellular uptake, solubility, and overall pharmacokinetics.\(^\text{48}\) Dextran-coated iron oxide nanoparticles are excellent non-toxic and biodegradable supports that have found uses in the preparation of multifunctional probes.\(^\text{49}\) Hilderbrand \textit{et al.} covalently linked \textbf{Oxazine 1} to a dextran-coated iron oxide nanoparticle via a 5-xopentanoate linking unit to create a probe \textbf{15} for the in vivo sensing of reactive oxygen/nitrogen species (ROS/RNS).\(^\text{50}\) As shown in Figure 1.15, in the presence of ROS/RNS the nanoparticle to dye linking unit is oxidatively cleaved releasing the free fluorescent compound \textbf{Oxazine 1}.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{PET_quenching.png}
\caption{PET quenching mechanism of NIR potassium sensing}
\end{figure}
Fluorescence imaging has been used in the medical field as a noninvasive method to visualize tissues in the body. Near infrared (NIR) fluorophores are of particular advantage than visible light probes because they allow for visualization without competing with absorption and autofluorescence from other macromolecules and water in the visible light spectrum. Our lab has adapted Oxazine 4 as a NIR fluorophore in the tissue specific illumination of the brachial plexus and sciatic nerve (Figure 1.16). To our knowledge, this work represents the first example of a small molecule nerve specific NIR fluorophore.
Gremlich et al. developed the NIR oxazine fluorophore AOI987 capable of penetrating the blood brain barrier and bind to amyloid plaques for their in vivo detection. The buildup of amyloid plaque in the brain has been associated with Alzheimer’s pathogenesis (Figure 1.17). The non-invasive detection and monitoring off the plaques can offer an avenue to further study its association to the disease to improve treatment methods.
Figure 1.17 In vivo fluorescence imaging of amyloid plaques with AOI987 in mice model by Gremlich et. al (reprinted from reference #52)

1.4.1.2 Benzophenoxazine

Analogous to phenoxazinium, benzo/a/phenoxazinium dyes have seen their shared popularity as biological sensors. Appending groups at their donor/acceptor sites that can be protonated/deprotonated at different pH levels, resulting in fluorescence change, has served as a good detection method to track intercellular pH changes. Ihara et al. reported the incorporation of N-substituted pyridinyl groups on the receptor of Nile blue to design a water soluble probe 16 for intercellular pH sensing (Figure 1.18).54 Similar probes were designed by Zhan et al. by attaching various electron donating phenyl moieties to the scaffold to yield a pH activable lysosome probe.55
Figure 1.18 Reversible Near-Infrared pH Probes based on benzo[a]phenoxazinium

A pH-responsive $N$-(-ethylated)pyridiniumylbenzo[a]phenoxazine dye $\text{BPox}$ was utilized by Chen et al. in the design of an albumin-based nanoprobe for the ratiometric photoacoustic imaging of tumors. Photoacoustic spectroscopy, the measurement of the sound waves generated during the thermal relaxation of an excited probe, can be used in compliment to fluorescence for enhanced biological imaging. The benzophenoxazine dye along with another pH unresponsive NIR dye $\text{IR825}$, used in this experiment as an internal reference, were encapsulated into human serum albumin (HSA) to form a nanoparticle $\text{HSA-BPox-IR825}$ that could penetrate the hydrophobic barriers of biological tissues for an accurate pH responsive imaging of tumors (Figure 1.19).
A near infrared benzophenoxazinium based probe 17 was specially engineered to contain an \textit{o}-phenylenediamine group that could undergo intramolecular cyclization in the presence of sodium nitrite to form compound 18. Structural change of the reactive \textit{o}-phenylenediamine group of the probe into a triazole ring in the presence of nitrite species ring elicited PET quenching, leading to fluorescence increase of the probe used for the selective in cellular imaging of exogenous nitrite (Figure 1.20).\textsuperscript{58}
Fluorescent dyes can be incorporated onto surfaces of material such as glass or optical fiber to create surface sensor for an array of applications. The fluorophore Oxazine 170 perchlorate was coated onto ethyl cellulose to produce a thin membrane used in the detection of ammonia in different environments.\textsuperscript{58-59}

Some oxazine analogs have also been evaluated in the structure inherent targeting of specific tissues. Our group has recently assessed and shown the ability of some oxazine analogs to specifically target tissues to be used as contrast agent in NIR image guided surgery. We demonstrated the superior tissue uptake of Oxazine 170 to several other analogs and proved it to be a successful fluorophore in highlighting thymus tissues in mice and pig models.\textsuperscript{60}
1.4.2 **Therapeutic application**

1.4.2.1 **Photosensitizer**

Photodynamic therapy (PDT) has garnered attention in medicine as a versatile treatment method for various medical conditions such as bacterial, fungal, viral infections, and in the fight against cancer. In PDT, a photosensitizer (PS) localized at the site of treatment is exposed to light to undergo series of electronic transitions which elicit toxicity in the micro environment through the generation of reactive oxygen species, resulting in death of the targeted cell as illustrated in Figure 1.23.\(^1\) An important feature of PDT is the ability of the photosensitizer to effectively

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**Figure 1.22** Color image, NIR fluorescence, and a merged image of the two of thymus tissue in mice stained using **Oxazine 170** contrast agent, by Wada et al. (reprinted from ref. 60)
associate with the malignant target, to avoid the obliteration of healthy sites during light treatment. Some oxazine dyes have been known to selectively distinguish malignant from healthy tissue in animals and have been investigated for their photosensitizing efficacy in the treatment against cancer. Oxazine dyes have also been explored as photosensitizer in the Antimicrobial Photodynamic Therapy (APDT) of localized infections. Another photosensitizing application of oxazine involves their use in the treatment of skin-tropic strains of Leishmania.

![Diagram of ROS generation in photodynamic therapy](image)

**Figure 1.23** Illustration of the generation of ROS in photodynamic therapy (PDT).

### 1.4.2.2 Antimalarial

A thorough review of literature reveals that prior to 2005, very few examples existed of the development of oxazine dyes as small molecule therapeutic agents. The few cases were isolated assessments of commercially available analogs, typically without further synthetic development to expand the analog set. As a result, for years the pharmacological potential of oxazine dyes remained vastly unexplored. Very recently, some oxazine dyes functionalized with alkyl, heterocyclic, and aromatic substituents were evaluated as anti-malarial agents. Their use as antimalarial laid on the delocalized lipophilic cation (DLC) hypothesis in which hydrophobic cations containing delocalized π-electrons traverse the cellular membrane into the cytosol and
accumulate inside organelles under a negative transmembrane potential such as the mitochondria, and digestive vacuoles to inhibit metabolic activity of the parasite. Their resurgence in the fight against malaria has prompted focus in the synthetic exploration of their architectures, to which several lead molecules with promising activities against chloroquine resistant plasmodium strains have been developed (Figure 1.24).67-71

**Figure 1.24** Illustration of the accumulation of DLC inside the mitochondria under negative membrane potential.

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**Figure 1.25** Oxazine dyes explored as antimalarial.
2 SYNTHESIS AND PHOTOPHYSICAL PROPERTIES OF PHENOXAZINE ANALOGS DESIGNED AS POTENTIAL ANTIMALARIALS

2.1 Introduction

Malaria remains amongst the deadliest infectious diseases plaguing developing countries with tropical climates. Although tremendous strides have been made in the treatment of malaria, the resurgence of drug resistant parasitic strains has refocused scientific efforts to enhance the potency of known therapeutics and the druggability of existing targets. It is often these can occur in a synergistic manner, and medicinal chemists have employed delocalized lipophilic cations (DLCs) with intrinsic fluorescence and organelle targeting properties as an emerging strategy in the fight against malaria.

Oxazines have garnered interest from scientists in both academia and in the industry as a useful developmental scaffold. When sandwiched between two phenyl rings, a rigid tricyclic system can be created that tend to be characterized by strong chemical stability and increased lipophilicity. Such system has seen its use in the building or as active substituents in molecules exhibiting many interesting biological properties. Phenoxazininiums are cationic analogs of 1,4-oxazine with two adjacently fused benzenes containing an amine and iminium moiety para to the central nitrogen (Figure 2.1). A survey of literature reveals that many phenoxazinium analogs exhibit desirable photophysical properties such as strong absorption of visible light and high fluorescence quantum yield which have fixated their use as fluorescent probes for an array of applications. The utility of the phenoxazinium scaffold is not only limited to its photo applications. It has also seen its use in developing small molecules therapeutic agents in the treatment against various infections. Phenoxazininiums have been explored in the development of small molecule ligands in the treatment
of chloroquine resistant malaria; however, such cases have mostly featured the assessment of commercially available analogs, typically without further synthetic development for structure-activity relationship (SAR) studies. This is perhaps because of synthetic challenges encountered during their analog derivatization.

![1,4-Oxazine, Phenoxazine, and Phenoazinium Dye](image)

**Figure 2.1** Scaffold of oxazine, phenoxazine, and phenoazinium dye.

Previously, our group has described the effect of appending alkyl substituents on the amines of the phenoazinium scaffold on its optical performance and biological uptake. This approach gave insight of the effect of lipophilicity on the optical profile and binding abilities of this class of compound, and the remarkable ability of several dyes with short alkyl chains to specifically target and bind to nerve, pancreas, and thymus tissues (Figure 2.2).51, 60 Very recently some phenoxazine and benzophenoxazine analogs functionalized with alkyl, heterocyclic, and aromatic substituents were evaluated for their anti-malarial properties to which several molecules with short chain, cyclic, and aryl amine moieties were shown to have promising activities against chloroquine resistant plasmodium strains.68-71

![Figure 2.2 Prototype Phenoxazine Probes for Thymus, Nerve, and Pancreatic Imaging](image)
Many of the analogs assessed as antimalarial bear skeletal resemblance to those used in our studies. Account for both designs, we sought to mimic trends found in the design of analogs with good antimalarial activities in literature with those found in the preparation of analogs with enhanced biological uptake properties to design and construct systems with good intrinsic fluorescence and improved antimalarial potency. In this work, phenoxazinium and benzophenoxazinium dyes containing short chain, rigid and cyclic amines were synthesized as an effort to enhance their use in the treatment of malaria. Phenoxazinium analogs containing rigid/cyclic moieties incorporated into the extremities of their chromophoric systems represent an exotic analog set that unfortunately, to date, only a handful have been reported in the literature. In this context, this work also introduces an efficient path for the diversification of the phenoxazinium scaffold.

2.2 Project Rationale

![Modulation of pi delocalization](image)

**Figure 2.3** General modifications to the phenoxazinium scaffold

The efficacy of small molecule therapeutic methods where an intracellular receptor is (believed to be) targeted often lies in the ability of the ligand to traverse the cell’s first barrier of protection, the plasma membrane. The plasma membrane consists of a hydrophobic phospholipid bilayer and
proteins that allow for the selective diffusion of substances in and out of the cell. Medicinal chemists have described predictive factors that can influence favorable diffusion of small molecules across the plasma membrane in the Lipinski’s “Rule of 5” such as polarity, size, and lipophilicity. Phenoxazinium are lipophilic compounds that can traverse the plasma membrane due to their distributed hydrophobic surface area as a result of the delocalization of pi electrons across the conjugated molecule. Once in the cytosol, they can specifically accumulate within organelles under negative potential because of their cationic nature.

The mechanism of action and factor governing the efficacies of phenoxazine in the treatment against malaria remains unclear, as it is the case with most potent antimalarials; however, the efficacy of the treatment methods have been recently correlated to the ability of the compounds to accumulate inside the membranes of organelles under a negative potential such as the mitochondria, digestive vacuoles, and inside the plasmodium specific organelle, the apicoplast, which can disrupt cellular activities in the parasitic cells, leading to their death. In order to enhance the efficacy of this treatment, we have synthesized a focused library of systematically modified substituted phenoxazinium analogs taking into account plasma membrane penetration and related antimalarial “drug like” design to begin speculating factors modulating their antimalarial potency.

We have synthesized compact analogs with short chained alkyl functionalities at multiple positions on the scaffold in the hope to enhance the cellular uptake functions and discourage promiscuous interaction with macromolecules during cellular distribution while working to maintaining their antimalarial potency (Figure 2.4). We have also prepared analogs with a fused phenyl to increase the delocalization and the hydrophobic surface area. Several analogs containing
the fused phenyl were equipped with a hydroxy group to increase water solubility and to serve as a handle for potential bioconjugation.

**Figure 2.4** Flexible, bridged, and cyclic phenoxazine and benzo[a]phenoxazine library

2.3 Experimental Overview

2.3.1 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. DMSO was obtained from Acros Organics (99.9%) (Belgium) and methanol was obtained from Sigma-Aldrich (≥ 99.9%) (St. Louis, MO). 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 Shimadzu RF-5301 Spectrofluorometer (Shimadzu Europe Inc.) 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 with a path length of 1 cm.
2.3.2 Preparation of N-alkylated-3-aminophenol

![Chemical Structures](image.png)

**Figure 2.5** Synthesis of N-alkylated 3-aminophenols

All the straight chain alkylated compounds 27a-b were prepared by dissolving 3-aminophenol in DMF, adding K₂CO₃, and rapidly heating the reaction flask to 70 °C. The alkyl halides were all liquids and were added dropwise for 15-30 min. Reactions were monitored by TLC until completion. Reaction workup proceeded by first removing DMF through serial extraction with ethyl acetate and water. The combined organic fractions were concentrated under reduced pressure and purified using column chromatography on normal phase silica and eluting with various compositions of Hexanes/AcOEt.

For the synthesis of compounds 28a-d, 3-aminophenol or m-anisidine (1.0 mol) was dissolved in dried toluene. Triethyl amine (2.3 mol) and Na₂CO₃ (2.5 mol) were added to the solution, followed by the addition of the 1,4-dibromobutane 28a-b or 1,5-dibromo pentane 28c-d.
(1.3 mol) dropwise over a period of 45-60 min. The reaction flask was heated at 110 °C under nitrogen o.n. Reactions were monitored by TLC until completion. Reaction workup proceeded by decanting the reaction liquid and extracting with water/DCM to remove the salts. The combined organic layers were concentrated under reduced pressure and purified using column chromatography on normal phase silica and eluting with various compositions of AcOEt/Hexanes.

Cyclic aminophenol 29 was prepared by reacting 3-aminophenol (1.0 mol) with 1,4-dibromobutan-2-ol (1.2 mol), and potassium carbonate (1.1 mol) in distilled water in a crimp-sealed thick-walled glass tube equipped with a micro-magnetic stirrer. The reaction tube was placed in a CEM Microwave Synthesis System, operated at 120 °C for 45 min with a power 150 Watt. After completion of the reaction, the organic portion was extracted into ethyl acetate. Removal of the solvent under reduced pressure and flash column chromatography using with various compositions of AcOEt/Hexanes as eluent afforded the pure product as an off-white solid.

Compound 30 was prepared by heating 3-aminophenol (1 mol) and bis(2-chloroethyl)amine (1.2 mol) in sulfolane at 150 °C o.n. Reaction progression was monitored by TLC until completion. Reaction workup proceeded by the dilution of the reaction in cold acetone. The reaction was allowed to stir in cold bath for an additional hour, to form a precipitate. The collected crystals were washed with acetone, dried, and recrystallized from methanol to yield the pure product as off-white solids.
2.3.3 *Synthesis of nitrosylated intermediates*

**Figure 2.6** Nitrosation of N-alkylated 3-aminophenols (compounds were used without purification)

All nitrosation reactions were conducted in the same manner as shown in Figure 2.6. An alkylated aminophenol (1.0 mol) was dissolved in EtOH and a molar excess of HCl was added. The entire system was cooled on a cold bath. Sodium nitrite (1.2 mol. eq.) was dissolved in the minimal amount of DI water and very carefully added dropwise to the reaction flask over 30-45 min. The reaction was allowed to stir for an additional 2-3 h on the ice bath. The precipitate was washed with water, ultra-sonicated in Et₂O, and filtrated to remove inorganic salts to give nitroso compounds 31-34, which were used in the next step without further purification.
2.3.4 Synthesis flexible and cyclic benzo[a]phenoxazinium dyes.

The benzo[a]phenoxazinium derivatives were prepared by refluxing the nitroso precursors (1.0 mol) with 1-naphthalamine (1.0 mol) for dyes 20, 21, 22 or with 5-Amino-2-naphthol (1.0 mol) for dyes 19a-b, 21, and 22a in IPA with hydrochloric or perchloric acid as shown in Figure 2.7 using a Dean Starks apparatus. Dye formation was visible with color change to dark blue.
occurring within the first hour of heating. The reaction was monitored by UV-Vis and halted when there was no change in its absorption spectra at ~600nm. The cooled reaction mixtures were concentrated under reduced pressure. The dark residue was then dissolved and ultra-sonicated in ethanol, precipitated with diethyl ether, and purified using flash column chromatography using various compositions of AcOEt/Hexanes or MeOH/DCM as eluent to afford the pure product as dark blue solids.

2.3.5 Preparation of diazonium salt (Diazotization)

Diazotization starts with a three-step conversion of acetonilide to \( p \)-nitroaniline, Acetonilide was added to a 50% solution of nitric acid and sulfuric acid on an ice bath with stirring for 30 min and allowed to reach room temperature. The reaction was quenched over ice and allowed to stir for an additional 20 min. The precipitate was collected, washed with water, and recrystallized from EtOH to yield pure \( p \)-nitroacetonilide as creamed colored solids. To an aqueous solution of \( p \)-nitroacetonilide under stirring, concentrated HCl was added dropwise for 30 min and heated under reflux. The reaction was cooled to r.t, quenched over ice water, and basified to pH 5.0. The precipitate was collected, washed with water, and dried in vacuo to yield pure yellow \( p \)-nitroaniline. \( p \)-Nitroaniline (1.0 ml) dissolved in a solution of concentrated hydrochloric acid and water was cooled in an ice-bath and sodium nitrite (1.0 mmol) dissolved in water was added dropwise. The reaction mixture was stirred at 0-5°C for an additional 20 min which turned to an orange color, signaling the formation of the diazonium specie that was used \textit{in situ} in the azo coupling reaction without further purification by adding a methanolic solution of 8-aminoquinoline, or 1-naphthalamine to the mixture at room temperature. The precipitate was filtered, washed with cold ethanol, and recrystallized from 2-propanol to yield the diarylazo intermediate 35.
2.3.6 *Azo coupling and synthesis of bridged benzophenoxazinium analogs*

Equimolar amount of diarylazo aromatic intermediate 37 and bridged aminophenol were dissolved in dried ethanol, and heated overnight at 75 °C. Reaction progression was monitored by TLC until completion. The reaction mixture was concentrated under reduced pressure, and purified by ultra-sonication in ethanol and diethyl ether, and purified using flash column chromatography using various compositions of AcOEt/Hexanes or MeOH/DCM as eluent to afford the pure product 23 and 24 as dark blue solids. The structure of dyes and the intermediates were confirmed by $^1$H NMR, $^{13}$C NMR (Appendix).
2.3.7 **Spectroscopic studies of dyes**

2.3.7.1 **Determination of molar absorptivity**

Serial dilutions of each dye were prepared in ethanol with concentrations ranging from $0.2 \times 10^{-5} - 1.60 \times 10^{-5}$ M. The maximum wavelength of absorption ($\lambda_{\text{abs}}$) 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.

2.4 **Results and Discussion**

2.4.1 **Synthesis of compact and bridged analogs**

The synthesis of phenoxazine 25f and benzo[a]phenoxazine 19-22 were achieved through the condensation reaction of a nitrosylated 3-(alkylatedamino)phenol with the corresponding 3-(alkylatedamino)phenol lacking the nitroso group, naphthalamines, or with 5-aminonaphthalen-2-ol, respectively. The synthesis begins with preparing alkylated aminophenols. The general method to obtain alkylated aryl amines is through the direct alkylation of aryl amines with alkyl halides. The alkylation reaction for analogs containing straight chain alkyl groups were conducted using equimolar amount of commercially available iodomethane or iodobutane and 3-aminophenol and doubled the molar amount of potassium carbonate in DMF at 70 °C.

Dialkylation of 3-aminophenol presented a challenge as primary amines are good nucleophile that when reacted with short chain alkyl halide form a secondary amine, which becomes an even better nucleophile than its predecessor. The secondary amine can react with excess alkyl halide to form a tertiary amine, which is also more nucleophilic than the previous two species, continuing the alkylation train until the subsequent formation of the fully alkylated quaternary amine/ammonium salt. This could be observed experimentally on the TLCs of the reaction progression showing the consumption of the starting reagents and the emergence of multiple
product spots corresponding to the secondary, tertiary and their salts, as well as the quaternary ammonium salt.

![Diagram showing basicity and nucleophilicity of substituted aryl amines](image)

**Figure 2.10** Arbitrary scale of basicity and nucleophilicity of substituted aryl amines.

Iodide has been widely used to enhance the reactivity of amines with certain electrophiles in SN<sub>2</sub> nucleophilic substitution reactions. Alkyl iodides often tend to decompose during storage when exposed to light, or in sealed reaction tubes at elevated temperatures, which can contribute to the decrease of the efficacy of the dialkylation reaction. To promote the formation of the product based on the molar ratio of the alkylating agent and aryl amine, catalytic amount of potassium iodide was added to the reaction mixture. The addition of potassium iodide favored the formation of the desired substituted amines perhaps by activating and enhancing the reactivity of the iodoalkane, or by stabilizing the salts of the major product to make the reaction remarkably faster and more effective. The reaction work up consisted of several washes with cold water to remove base and some of the quaternary salts from undesired alkylation, followed by a series of extractions from cold water and AcOEt to remove DMF. The oily residues were then taken up in ethanol, filtered to remove salts, and then loaded onto silica for purification using normal phase silica and ethyl acetate/hexanes solvent systems.
The cyclic aminophenol 28 were prepared following published procedures involving the reaction of 3-aminophenol with vicinal dibromoalkane of varying length, depending on the size of the cyclic moiety desired, in the presence of TEA in toluene under nitrogen gas. After TLC showed full consumption of the starting reagents, workup of the solvent involved the decanting of the liquid from the reaction mixture, which was filtered to remove any solids, and extracted with water/DCM. The combined organic layers were concentrated under reduced pressure and purified using column chromatography on normal phase silica and eluting with various compositions of AcOEt/Hexanes. Following similar protocol for the synthesis of compound 29 showed little reaction progression after 2 hr when monitored using TLC and a smorgasbord of products on TLC when allowed to react overnight under inert atmosphere. For this reaction, microwave conditions were adopted and proved to be superior to the conventional methods in attaining satisfactory yields. Water was used as both solvent and reaction promoter without the need for base. The product was isolated through extraction with ethyl acetate and purified using column chromatography on normal phase silica and eluting with various compositions of AcOEt/Hexanes.

The next step in the formation of the final product consisted of the nitrosation reaction of the alkylated aminophenols following published procedures. Concentrated HCl was added to an ethanolic solution of the starting material placed in an iced bath. Sodium nitrite (1.2 mol. eq.) dissolved in minimal amount of water was added slowly over 30-45 min. The reaction was maintained at cold temperature and allowed to stir for 3 hours. If no precipitate formed, the reaction was placed in a fridge overnight. The precipitate was collected, washed with water, ultra-sonicated in Et₂O, and filtrated to remove inorganic salts to give the nitroso compounds. Special care was taken to maintain the compound at cold temperature, as heating above room temperature leads to
the decomposition of the compound. The nitroso compounds were used without further purification.

Dye formation was achieved through the condensation reaction of equimolar amount of the nitroso precursors (1.0 mol) with aminophenol, or 1-naphthalamine, or with 5-Amino-2-naphthol for the formation of phenoxazine, or benzophenoxazine and their phenol analogs, respectively. The reaction was conducted in IPA with hydrochloric or perchloric acid as shown. A Dean Starks apparatus was used to remove water formed during the reaction to drive forward the cyclization into the dye. Dye formation was visible with a color change of the solution to dark blue occurring within the first hour of heating. The reaction was monitored by UV-Vis and halted when there was no further change to the ratio of the peaks observed on the absorption spectra. The cooled reaction mixture was concentrated under reduced pressure. The dark residue was then dissolved and ultrasonicated in ethanol, precipitated with diethyl ether, and purified using flash column chromatography with various compositions of AcOEt/Hexanes or MeOH/DCM as eluent, to afford the pure product as dark blue solids.

Fused analogs were synthesized following a slightly altered arylazo mediated route. Bridged aminophenols do not readily convert into the desired nitroso intermediates for subsequent cyclization despite the presence of a hydroxy ortho/para directing group, therefore this route could not be explored for the synthesis of the desired fused benzo[a]phenoxazine analogs. Literature reports one example of the synthesis of analogs of this bridged class. It employs an arylazo mediated route instead of the common nitroso intermediate. Alternatively, compounds 23, and 24 were prepared following the azo method which start by the preparation of p-nitroaniline which is then converted into the diazonium salt through the nitrosation of the amine with nitrous acid (generated in situ from sodium nitrite and sulfuric acid). Diazonium salt are unstable product that
were used in the next step without further purification. The diazonium salt was reacted in situ with the aminophenol to form an electronically activated aminophenol. Heating of the diarylazol with the corresponding bridged aminophenol in DMF in the presence of percholric acid lead to the formation of the dyes, shown by appearance of a new peak at ~640 nm on the UV-vis spectrum, characteristic of phenoxazinium dyes. The following method was found to be well suited to introduce bridged amino moieties into the benzophenoxazine architecture but the yields were exceptionally low, judged from the intensities of the dye to starting material peak on UV-Vis (7%). Reaction conditions were tweaked replacing DMF with dry ethanol as solvent, and heating the mixture under reflux condition for 1 hr under inert atmosphere, after which the reaction was allowed to stir at r.t for another 5 hr. The reaction mixture was concentrated under reduced pressure, and purified by ultra-sonication in ethanol and diethyl ether, and purified using column chromatography on normal phase silica and eluting with various compositions of AcOEt/Hexanes and DCM/MeOH.

2.4.2 Characterization

Compound 22b was chosen as a representative for complete chemical characterization and analysis. \(^1\)H NMR spectra measured in DMSO-\(d_6\) is shown in Figure 4 with color coded boxes matching the peaks to protons on the structure. The protons on the carbon of the piperazine ring appear as doublets in the aliphatic region (3.64-3.26 ppm) with an integration of four each. The rest of the signals appear in the aromatic region of the spectrum. Most evident being the OH- proton appearing as a singlet at 11.2 ppm, followed by the iminium protons appearing as a doublet at 10.37-10.25 ppm, this shift due to the due to the conjugation of the molecule. Amine and HCl protons of piperazine are both shifted downfield because of the
presence of the HCl. The rest of the peaks appear in the range of 6.8-8.4 with their respective splitting and integrations. Solvent and water peaks are visible at 2.5 ppm and 3.2 ppm.

![Image of NMR spectrum](image)

**Figure 2.11** Figure 2.11 1H NMR spectrum of compound 22b

$^{13}$C NMR spectrum of compound **22b** displays 16 peaks from 97.3-162.9 ppm corresponding to the 16 aromatic carbons and two peaks at 46.2 ppm and 42.7 ppm corresponding to the four carbons on the piperazine ring, appearing as two equivalent carbons.
2.4.3 Photophysical and physicochemical properties of analogs

In order to determine the effect of the $N$-alkylation of the dyes on their optical profiles, the optical properties of all dyes were conducted in EtOH and compared to commercially available Nile blue. The addition of a hydroxy group on the phenyl group increased solubility, increasing the molar absorptivity of the compound by $\sim 20,000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for dyes 19a-b. Addition of cyclic alkyl group did not significantly affect the absorbance, but the stoke shift for the compounds were lowered by a half and by a quarter respectively for dyes 20 and 21 compared to NB. The two bridged compounds 23 and 24 had blue shift absorption $\lambda_{\text{max}}$ values from 5-30 nm. Compound 22b
had the lowest absorbance value and highest stokes shift of all compounds in this series and featured the most water-soluble groups.

<table>
<thead>
<tr>
<th>Dyes</th>
<th>( \lambda_{\text{abs}} )</th>
<th>( \lambda_{\text{em}} )</th>
<th>Stokes Shift (nm)</th>
<th>( \varepsilon ) (L·mol(^{-1} )·cm(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>630</td>
<td>660</td>
<td>30</td>
<td>76,800</td>
</tr>
<tr>
<td>19a</td>
<td>615</td>
<td>654</td>
<td>39</td>
<td>87,520</td>
</tr>
<tr>
<td>19b</td>
<td>620</td>
<td>660</td>
<td>40</td>
<td>99,430</td>
</tr>
<tr>
<td>20</td>
<td>620</td>
<td>668</td>
<td>48</td>
<td>44,000</td>
</tr>
<tr>
<td>21</td>
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<td>22,110</td>
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<td>665</td>
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<tr>
<td>22b</td>
<td>600</td>
<td>659</td>
<td>59</td>
<td>42,140</td>
</tr>
<tr>
<td>23</td>
<td>635</td>
<td>646</td>
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<td>24</td>
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<tr>
<td>25f</td>
<td>645</td>
<td>658</td>
<td>13</td>
<td>17,000</td>
</tr>
</tbody>
</table>

Table 2.1 Optical properties of dyes 19-25f

![Absorbance spectra of compounds 19-25f.](image)
The physicochemical profile of small molecules is very important in their design as potentially bioactive ligands, especially in a ligand-based design where definitive information on a binding target or receptor is not available but a scaffold of interest is known. Physicochemical assessment allows for the understanding of the molecular character of analogs of a scaffold such as their molecular weight; Log D; numbers of H-bond donors (HBD) and H-bond acceptors (HBA), which are determinants of the interaction of the ligands with macromolecules once inside the body and heavily influence their solubility and permeability. In medicinal chemistry, those factors are descriptors of the molecules’ bioavailability which is used as a primary descriptor to assess the “drug-likeness” of a scaffold- how those properties measure with those of approved drugs. Physicochemical calculation can be performed using modern software that are convenient to use. We evaluated the physicochemical profile of the synthesized phenoxazine derivatives using Chemaxon calculator plugin in Marvin using the Lipinski and Veber rules as the first net for fishing.
analogs with undesirable profiles from our selection pool and comparing them to previously synthesized antimalarial SSJ206 and MB. For the investigation of a hypothesized mode of action for this scaffold, we have also calculated the molecular surface area (MSA), and dipole moment (DM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>M.W</th>
<th>LogD</th>
<th>NH/OH</th>
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Table 2.2 Physicochemical evaluation of phenoxazine and benzophenoxazine analogs.
2.4.4 Structural assessment of the antimalarial “drug-likeness” of analogs

The design of the following phenoxazine and benzophenoxazine analogs as antiprotozoal were based off revisited trends found in the evaluation of commercially available tricyclic cations with amine bearing side chains. Methylene blue (MB) is a tricyclic cation previously coined the magic bullet for its ability to specifically stain and kill plasmodium parasite and other protozoal infections. The plasmodium parasite gains its virulence inside its human host during its blood stage by invading red blood cells and consuming hemoglobin as its main nutrient source, leaving behind hematin, where the heme’s iron is in its oxidized (+3) state which is responsible for the production of reactive oxygen species leading to oxidative stress to the parasite. Through evolutionary ingenuity, the malaria parasite has engineered a detoxification process crucial for its survival by forming insoluble aggregate of the toxic heme groups called hemozoin or malaria pigment. Although solid-state structural identification of hematin has not been achieved, there exist conclusive evidence that hemozoin is structurally identical to synthetic β-hematin which consist of strands of Fe (III)-porphyrin units, linked into a polymer by propionate oxygen-iron bonds. MB and many other tricyclic cationic molecules have been speculated to target the sequestration of hemozoin. They have been postulated to accumulate in the digestive vacuoles of malaria parasites and act by blocking the heme polymerization through:

1. directly binding heme monomers and preventing the polymerization of heme
2. inhibiting enzymatic processes involved in the polymerizing heme
3. or through the interaction and subsequent decrystallization of hemozoin in the digestive vacuole of P. falciparum

Methylene blue has been used to enhance the efficacy of known therapeutic malaria treatments through the non-competitive inhibition of Plasmodium falciparum glutathione
reductase (GR), a redox enzyme responsible for buffering intercellular oxidative stress, by retaining low glutathione levels inside the cytosol of malaria cells and in turn enhances the activity of some antimalarial agents such as chloroquine, dihydroartemisinin, and mefloquine.\textsuperscript{84}

Tricyclic cationic compounds with N-substituted alkyl chains have been found more active against malaria, perhaps because of increased lipophilicity which can enhance the molecules’ cellular diffusion. Once inside the cell, the molecules can invoke its antimalarial abilities through the multiple mode of actions plausible for its class. A review of recently publish studies conducting the evaluation of tricyclic cations related to MB as antimalarial reveals a general trend in the alkylation of the scaffold’s donor and acceptor nitrogen groups and their antimalarial potency. A carbon chain length of $\leq$3 on their amine has been described as the “sweet spot” to attain favorable lipophilicity while maintaining adequate antimalarial potency. Chain longer than 3 carbons have been shown to reduce the potency of the drugs. This is perhaps due to the steric blockade imposed by the flailing alkyl chain during the ligand to heme interaction. Another featuring that was discovered to enhance the drug profile of the scaffold against malaria was the addition of dimethyl group at their 2, 8 positions or a single methyl group at either position. A comparative biophysical study of the binding of two analogous tricyclic cations with lysozyme, a protein abundant in protective fluids of a lot of mammals, showed the superior ability of an analog containing a methyl group at the 8 position to the protein over the corresponding analog lacking a methyl group.\textsuperscript{83} We designed a series of compounds featuring small analogous changes that could be used to begin defining factors affecting their antimalarial potency. Compounds 25a-e containing N-alkylated amines of carbon length shorter than 3 with or without a methyl group at their 2 position were selected to investigate the effect of the structural modification on the antimalarial potency of the analog set.
We also designed and synthesized a derivative of a known phenoxazine analog **SSJ208** with good antimalarial activity (IC₅₀ = 0.006 μM) by adding a 2-hydroxy moiety to the cyclic amine to take investigate the postulated mechanism of action of hemozoin decrystallization by delocalized lipophilic cations containing amine bearing side chain and groups with the ability to donate electron proposed by Ulrich *et al.* The idea is that the ligand can interact with the aggregated hemozoin, hypothetically by binding the heme groups to the hydrophilic surface of the molecule through π-π stacking and aggregation due to its planar delocalized structure, while the iminium moiety of could interact with the carboxylate of the heme through electrostatic interaction, placing the hydroxy moiety in the crux of hematin’s iron to oxygen bridge. The Oxygen could act as a reducer of the Fe³⁺ of the bridge to Fe²⁺, in the process breaking the Fe-O bond and releasing the toxic hematin. Crystal structure and biophysical studies of the interaction of the molecule with heme should be conducted to support this hypothesis, upon antimalarial evaluation.

Benzo[a]phenoxazine derivatives were also designed with investigative features in mind geared towards enhancing antimalarial property. The phenyl ring was adapted onto the dye architecture to enhance the hydrophobicity and planarity of the dye required for adhesion onto the surface of the heme. We also maintained an unsubstituted iminium at the 7 position to investigate its effect on the antimalarial potency of the analog set. The presence of both fully substituted amine and primary imine on the scaffold means the presence of groups sensitive to changes in proton concentration, indeed Nile blue analogs are known to be pH sensitive dyes. The designed compounds are predicted to reversibly change from their protonated state to neutral to reversibly enter the parasite digestive vacuole (pKa ~3.7-6.5), the site of hemoglobin degradation. We previously concluded through an investigative study of the binding of alkylated Nile blue dyes with Human serum albumin (HSA) that mono substituted analogs did not bind at all to the protein
and that the di-substituted dyes showed a slight improvement in binding. HSA is the most common protein found in human blood plasma, and thus the most used mechanism for transporting drugs inside the body and delivery them to their target. The effective binding of a drug candidate to HSA can have a pronounced effect on drug efficacy, retention/clearance, and toxicity. We sought to included cyclic alkyl groups retaining the C ≤3 rule to enhance HSA binding of the compounds.

2.4.5 Biodistribution

One of the main drawbacks in the used of tricyclic cationic molecules is their ability to stain internal tissues. Another reason for designing benzo[a]phenoxazine analogs was to increase the lipophilicity of the molecules and encourage total clearance without organ staining. Analogs of the benzo[a]phenoxazine containing N-alkylated and varying cyclic amine and their 2-hydroxy bioisosteres were synthesized to increase clearance and decrease staining of organs. A selected member of the benzo[a]phenoxazine class containing a pyrrolidinyl group was selected for in vivo biodistribution studies in a mice model through our collaborator at the Massachusetts General Hospital and Harvard Medical School, shown in Figure 2.15 and 2.16. The imaging data shows that the compound cleared though all internal organs and accumulated in the small intestines through the gall bladder and biliary tract, signifying that the molecule is absorbed through the gut when administered, and has higher clearance and no profuse staining of tissues.
**Figure 2.15** Biodistribution imaging of compound 2a

**Figure 2.16** Gallbladder uptake of compound 2a
3 Conclusion

The oxazine core represents an attractive scaffold from which fluorescent probes of the phenoxazine and benzophenoxazine have been developed. The rational design of a focused library of polycyclic lipophilic cations was afforded through the synthetic derivatization the phenoxazine and benzophenoxazine with the principle aim of mimicking recent trends found in the development of potent antimalarial to cater to the enhancement of their “drug like” profile. This work presents 14 dyes and their photophysical and physicochemical properties, a structural assessment of their antimalarial “drug like” profile, and the biodistribution studies of analog 20. Such modification, in conjunction with the proven effectiveness of the scaffold, could lead to the development of effective antimalarial agents. The following libraries are currently being evaluated in vitro for their antimalarial efficacy against drug sensitive malaria strain NF5 and the multidrug-resistant strain K1, and for their antitrypanosomal properties against T. brucei cells. Their toxicity against mammalian cells will be studied to determine their selectivity.
4 Experimental

**Alkylation of 3-aminophenol.** 3-Aminophenol (45.82 mmol) was dissolved in DMF along with K$_2$CO$_3$ (2 mol. equ.). The mixture was sonicated and allowed to mix for 15 minutes. Alkyl halide (2 equ. in respect to 3-aminophenol) 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 chromatography eluting first with Hexanes. This was increased to 7:3 Hex/AcOEt to finish the separation. The middle spot was confirmed by spectroscopic analysis of the eluting products to be the desired compound.

**3-(dimethylamino)phenol.** Compound 27a. Yield 43%. $^1$H NMR (400 MHz, (CD$_3$)$_2$SO-d$_6$): δ 8.992 (s, 1H), δ 6.932 (t, $J$ = 8 Hz, 1 H), δ 6.155 (d, $J$ = 8.4 Hz, 1 H), δ 6.101 (s, 1 H), δ 6.079 (d, $J$ = 8 Hz, 1 H), δ 2.824 (S, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 100.00, 104.11, 104.25, 129.92, 152.29, 158.55.

**3-(diethylamino)phenol.** Compound 27b. Yield 38%. 1H NMR (400 MHz, (CD$_3$)$_2$SO-d$_6$): δ 8.914 (s, 1H), 6.892 (t, $J$ = 8 Hz, 1H), 6.095 (d, $J$ = 2.4 Hz, 1H), 6.078 (s, 1H), 5.987 (d, $J$ = 7.6 Hz, 1H), 3.248 (q, $J$ = 7 Hz, 4H), 1.054 (t, $J$ = 7 Hz, 6H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 25.22, 48.77, 19.06, 29.64, 43.94, 95.26, 120.05, 130.71, 144.29, 162.77, 166.70; m.p. 156-158 °C.

**3-(pyrrolidin-1-yl)phenol and 3-(piperidin-1-yl)phenol.** Compound 28a-d: A mixture of 3-aminophenol (1 equ.) or m-anisidine with 1,4-dichlorobutane (1.2 equ.) or 1,5-dichloropentane was refluxed in dry toluene for 24 h and then cooled to room temperature. Triethyl amine (2 equ.) and sodium carbonate (1 equ.) in 10 ml distilled water were added to the mixture. The reaction mixture was refluxed for another 24 h. After completion of the reaction, the solvent was reduced
and the organic portion was extracted with CH$_2$Cl$_2$. The solvent was evaporated and the crude product was purified using silica gel column chromatography with petroleum ether/ethyl acetate as the eluent to yield off white crystals.

**3-(pyrrolidin-1-yl)phenol. Compound 28a.** 45% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.967 (s, 1H), 6.909 (t, $J$ = 8 Hz, 1H), 6.021 (d, $J$ = 8Hz, 1H), 5.974 (d, $J$= 8Hz, 1H), 5.935 (s, 1H), 3.137 (t, 4H), 1.903 (t, 4H), 1.526 (m, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 25.35, 47.81, 99.24, 103.38, 103.71, 130.01, 149.39, 158.58.

**1-(3-methoxyphenyl)pyrrolidine. Compound 28b.** 74% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.917 (q, $J$ = 3.2Hz, 4H), 3.187 (m, 4H), 3.741 (s, 3H), 6.164 (q, $J$ = 6.4Hz, 1H), 6.236 (q, $J$= 5.6 Hz, 1H), 7.084 (t, $J$=8.4, 1H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 25.42, 31.44, 34.09, 47.73, 55.00, 98.01, 100.94, 105.15, 129.93, 149.41, 160.88.

**3-(piperidin-1-yl)phenol. Compound 28c.** 68% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 9.048 (s, 1H), 6.956 (t, $J$ = 8.2 Hz, 1H), 6.352 (d, $J$ = 8.4 Hz, 1H), 6.291 (s, 1H), 6.172 (d, $J$= 8Hz, 1H), 3.063 (t, $J$=5Hz, 4H), 1.583 (t, 4H), 1.520 (m, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 24.33, 25.62, 50.24, 103.42, 106.43, 107.63, 129.97, 153.21, 158.54.

**1-(3-methoxyphenyl)piperidine. Compound 28d.** 55% yield. $^1$H NMR (400 MHz, DMSO-d$_6$) δ 7.092 (t, $J$= 8.4 Hz, 1H), δ 6.488 (d, $J$= 2 Hz, 1H), δ 6.446 (t, $J$= 2.4 Hz, 1H), δ 6.346 (d, $J$= 7.5 Hz, 1H), δ 3.711(s, 3H), δ 3.097 (t, $J$= 5.2 Hz, 4H), δ 1.596 (m, 4H), δ 1.521 (t, $J$= 5.6 Hz, 1H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 25.47, 25.76, 50.04, 55.14, 102.32, 104.04, 108.97, 129.89, 153.50, 160.69.

**1-(3-hydroxyphenyl)pyrrolidin-3-ol. Compound 29.** 38% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.854 (m, 1H), δ 2.035 (m, 1H), 3.027 (t, 1H), 3.025-3.371 (m, 3H), 4.377 (s, 1H), 4.938 (s, $J$= 8 Hz, 1H), 5.923 (s, 1H), 5.964 (d, $J$= 8.4 Hz, 1H), 6.024 (d, $J$= 1.2 Hz, 2H), 6.925 (t, $J$= 1.2 Hz,
59

2H), 8.981 (s, 1H), $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 34.17, 45.85, 56.41, 69.76, 98.89, 103.18, 103040, 130.04, 149.49, 158.60.

3-(piperazin-1-yl)phenol hydrochloride. Compound 30. 35% yield. A round bottom flask charged with 3-aminophenol (1 equ.) and bis(2-chloroethyl)amine hydrochloride (1.3 equ.) and 20 mL of sulfolane was heated to 150 °C overnight, by which the time the reaction was completed as monitored by TLC. The reaction mixture was cooled to 45 °C and diluted with 50 mL of acetone and further cooled to 0°C. The mixture was maintained at 0°C for 1 more hour to precipitate the desired product, which was filtered and washed with chilled acetone and dried under vacuum to yield the pure product as light brown crystals. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.551 (s, 1H), 7.066 (t, $J$= 8.2, 6H), 6.500 (d, $J$ = 10.8Hz, 1H), 6.483 (S, 1H), 6.38 (d, $J$= 8, 1H), 3.382 (t, 4H), 3.22 (t, 4H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$

General procedure for the nitrosylation of alkylated 3-aminophenols. A solution of N-alkylated aminophenol dissolved in 1:1 ethanol/HCl was stirred in an ice bath. Over a period of 1 h, NaNO$_2$ (1 mol. eq.) in 2 mL of water was slowly added to the mixture dropwise. The solution was left to stir on an ice bath for the remainder of the reaction to maintain a temperature at 0-5 °C. Precipitate was collect by vacuum filtration, and then used without any further purification to avoid decomposition. In the cases where no precipitate formed, the reaction pH was adjusted to ~9 with K$_2$CO$_3$, and extracted with ethyl acetate. The combined organic layers were dried from magnesium sulfate and concentrated under reduced pressure to give the nitroso compound.

General Procedure for Synthesis of flexible, and cyclic phoxazinium dyes. Nitroso compounds were dissolved in in $i$-PrOH (10 ml) in the presence of a catalytic amount of HClO$_4$. A solution of compound the respective napthalamine in $i$-PrOH (10 ml) was added dropwise to the above mixture in 30-45 min and heated at 75 °C. The reaction was monitored via UV-Vis. After
completion, the dark blue solution was concentrated under reduced pressure. The residue was ultrasonic in ethanol/ether for 30 min and filtered to yield the dye. Some dyes were purified by column chromatography with silica gel, eluting with various concentration of Hex/AcOEt

**9-(dimethylamino)-2-hydroxy-5H-benzo/a phenoxazin-5-iminium perchlorate.** Compound 19a. 27% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.065 (s, 1H), 9.944 (s, 1H), 9.819 (s, 1H), 8.348 (d, $J$= 8.8 Hz, 1H), 8.064 (s, 1H), 7.726 (d, $J$= 9.2 Hz, 1H), z′ 7.261 (d, $J$= 3.7, 1H), 7.088 (d, $J$=8 Hz, 1H), 6.844 (s, 1H), 6.714 (s, 1H), 3.201 (s, 6H). 282-284 °C

**9-(diethylamino)-2-hydroxy-5H-benzo/a phenoxazin-5-iminium perchlorate.** Compound 19b. 18% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.184 (s, 1H), 10.310 (s, 1H), 10.027 (s, 1H), 8.409 (d, $J$= 8.8 Hz, 1H), 7.990 (s, 1H), 7.613 (d, $J$= 9.2 Hz, 1H), 7.222 (d, $J$= 8, 1H), 7.006 (d, $J$=9.2 Hz, 1H), 6.829 (s, 1H), 6.778 (s, 1H), 1.281-1.024 (m, 10H). 285-287 °C

**9-(pyrrolidin-1-yl)-5H-benzo/a phenoxazin-5-iminium perchlorate.** Compound 20. 13% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 2.041 (m, 4H), 3.494 (m, 4H), 6.673 (s, 1H), 6.755 (s, 1H), 6.973 (d, $J$= 7.2, 1H), 7.711(d, $J$= 8.8 Hz, 1H), 7.851 (t, $J$= 7.6, 1H), 7.959 (t, $J$=7.2 Hz, 1H), 8.401 (d, $J$=8, 1H), 8.702 (d, $J$=7.6, 1H), 9.741(d, 2H). (100 MHz, DMSO-d$_6$) δ 29.16, 48.54, 55.78, 58.25, 69.19, 70.24, 77.03, 96.75, 97.21, 117.80, 119.13, 133.84, 134.00, 134.69, 149.85, 155.37, 157.51, 166.85. 260-264 °C

**9-(piperidin-1-yl)-5H-benzo/a phenoxazin-5-iminium perchlorate.** Compound 21. 11% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 2.041 (m, 4H), 3.494 (m, 4H), 6.673 (s, 1H), 6.755 (s, 1H), 6.973 (d, $J$= 7.2, 1H), 7.711(d, $J$= 8.8 Hz, 1H), 7.851 (t, $J$= 7.6, 1H), 7.959 (t, $J$=7.2 Hz, 1H), 8.401 (d, $J$=8, 1H), 8.702 (d, $J$=7.6, 1H), 9.741(d, 2H). (100 MHz, DMSO-d$_6$) δ 29.16, 48.54, 55.78, 58.25, 69.19, 70.24, 77.03, 96.75, 97.21, 117.80, 119.13, 133.84, 134.00, 134.69, 149.85, 155.37, 157.51, 166.85. 260-264 °C
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 10.533 (s, 1H), 10.403 (s, 1H), 9.173 (s, 1H), 7.974 (t, $J$= 7.6, 1H), 7.405 (d, $J$= 2 Hz, 1H), 7.382 (d, $J$= 2 Hz, 1H), 7.291 (s, 1H), 6.949 (s, 1H), 7.828 (t, $J$=7.6 Hz, 1H), 7.511 (s, 1H), 3.880 (t, 4H), 3.287 (t, 4H). 291-293 °C

2-hydroxy-9-(piperazin-1-yl)-5H-benzo[a]phenoxazin-5-iminium perchlorate. Compound 22b. 11 % yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.230 (s, 1H), 10.374 (s, 1H), 10.250 (s, 1H), 9.245 (s, 1H), 8.417 (d, $J$=8.8 Hz, 1H), 8.135 (s, 1H), 7.873 (d, $J$= 9.2 Hz, 1H), 7.342-7.320(m, 2H), 7.262 (s, 1H), 6.808 (s, 1H), 3.846 (t, 4H), 3.262 (t, 4H). (100 MHz, DMSO-d$_6$) $\delta$ 42.75, 44.30, 97.38, 99.27, 109.87, 114.77, 115.76, 119.95, 127.96, 128.15, 132.16, 134.68, 138.52, 147.21, 152.19, 154.14, 162.84, 162.90.

(E)-1-(7-(dimethylamino)-3H-phenoxazin-3-ylidene)-3-hydroxypyrrolidin-1-ium chloride. Compound 25f. 7 % yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.230 (s, 1H), 10.374 (s, 1H), 10.250 (s, 1H), 9.245 (s, 1H), 8.417 (d, $J$=8.8 Hz, 1H), 8.135 (s, 1H), 7.873 (d, $J$= 9.2 Hz, 1H), 7.342-7.320(m, 2H), 7.262 (s, 1H), 6.808 (s, 1H), 3.846 (t, 4H), 3.262 (t, 4H). (100 MHz, DMSO-d$_6$) $\delta$ 108.74, 131.25, 132.16, 134.74, 135.16, 137.04, 147.21, 154.14, 162.84, 162.90.

General Procedure for the preparation of 4-arylazo-1-naphthylamines. A solution of the appropriate $p$-nitrophenyldiazonium (1 equ.) in aqueous sulfuric acid was added to a solution (100 mL) of the appropriate 1-naphthylamine or amino quinoline under stirring. After 1h, the cooled reaction mixture was neutralized with aqueous NaOH, and the precipitated product filtered off and recrystallized from n-butanol to yield pure 4-Arylazo-1-naphthylamines.

(E)-4-((4-nitrophenyl)diazenyl)naphthalen-1-amine. Compound 35. 78 % yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.924 (d, $J$= 8.4 Hz, 1H), 8.376 (d, $J$= 2 Hz, 2H), 8.254 (d, 1H), 8.061 (m, 3H), 7.693 (t, $J$= 7.4 Hz, 1H), 7.548 (m, 3H), 6.820 (d, $J$= 8.8 Hz, 1H). (100 MHz, DMSO-d$_6$) $\delta$ 108.74,
General Procedures for the preparation of bridged phenoxazinium Dyes. A mixture respective aminophenol and the appropriate 4-arylazo-1-naphthylamine dissolved in IPA (50 mL) containing perchloric acid was refluxed at 85 °C. Reaction progression was monitored using TLC, and UV-Vis spectroscopy until completion. After cooling to room temperature the reaction mixture was concentrated under reduced pressure, the product was ultra-sonicated in 5ml of ethanol, filtered off to give the pure dye. In some cases, it was found necessary to purify the product by column chromatography on silica gel, using various concentrations of Hex/AcOEt as the eluent.

**9,10,11,12-tetrahydro-5H-benzo[a/pyrido[2,3-i]phenoxazin-5-iminium perchlorate.**

Compound 23. 56 % yield. ¹H NMR (400 MHz, CDCl₃) δ 9.453 (s, 2H), 9.089 (s, 1H), 8.797 (d, J = 8 Hz, 1H), 8.453 (d, J= 8, 1H), 7.974 (t, J= 7.6, 1H), 7.853 (t, J=7.6 Hz, 1H), 7.660 (s, 1H), 6.840 (s, 1H), 6.711 (s, 1H), 3.493 (t, 2H), 2.912 (m, 2H), 1.906 (m, 2H). (100 MHz, DMSO-d₆) 159.89, 154.74, 154.72, 151.00, 147.71, 132.47, 313.92, 131.52, 131.23, 129.48, 126.36, 124.38, 124.11, 122.76, 96.87, 96.39, 41. 64, 26.62, 20.18. 325°C decomposed

**2,3,6,7-tetrahydro-1H,5H,14H-benzo/a/quinolizino[1,9-hi]phenoxazin-14-iminium perchlorate.** Compound 24. 50 % yield. ¹H NMR (400 MHz, CDCl₃) δ 9.259 (s, 2H), 8.760 (d, J= 8Hz, 1H), 8.427 (d, J = 8 Hz, 1H), 7.974 (t, J= 7.6, 1H), 7.828 (t, J=7.6 Hz, 1H), 7.511 (s, 1H), 6.826 (s, 1H), 3.591 (t, 4H), 2.892 (m, 4H), 1.982 (m, 4H). (100 MHz, DMSO-d₆) 159.07, 151.01, 150.71, 143.45, 132.14, 131.79, 331.48, 130.71, 129.14, 128.98, 126.36, 127.16, 124.25, 124.03, 122.59, 105.47, 51.26, 50.75, 27.21, 20.48, 19.43, 19.29. 278-281°C
REFERENCES


APPENDICES

Appendix A
3-pyrrolidinyl-anisole
Exact Mass: 310.16
m/z: 310.16 (100.0%), 311.16 (19.5%), 312.16 (1.8%), 311.15 (1.1%)