A Red Light at the End of the Tunnel: Towards Optimal Red-Shifted Azobenzenes

by

Kareem Jarrah

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Chemistry
University of Toronto

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2017

Abstract

Azobenzenes have been used for almost half a century to impart spatiotemporal control on biological systems by way of an external stimulus - light. A limitation of previously reported azobenzenes is the requirement for ultraviolet to blue light to promote trans-to-cis isomerization. Light in this range of the spectrum poorly penetrates tissue and is cytotoxic, limiting in vivo applications. Through design and synthesis of an azobenzene derivative with a fused dioxane ring and a methoxy substituent on each phenyl ring, we obtained a core structure with a bathochromically-shifted absorption spectrum. Further substitution with para pyrrolidine moieties raised the pKₐ of this azobenzene such that a portion is protonated at physiological pH allowing it to absorb at wavelengths in the near-infrared region. The compound undergoes trans-to-cis isomerization with 720 nm light and relaxes back to the trans isomer with a half-life on the order of 1 s.
Acknowledgments

Long ago, I believed success – however it may be defined - was the product of a person’s hard work. As I continue to learn, I increasingly see that success is only possible with the love and support of family and friends.

Occasionally, experiments fail and work becomes difficult, but no matter the hour and no matter the issue, my family was always by my side. To my mother, who first inspired my love for science and continually keeps my moral compass pointed North, I am eternally grateful. My sister, Mona, supported and helped me more than anyone else could, by shouldering more than she should ever have to. To these two strong women, I owe so much and I hope I can one day repay their patience, generosity, and immeasurable kindness.

Very often we take those closest to us for granted, as their presence is so ubiquitous in our lives. Indeed I have been guilty of this, but I would like to acknowledge the amazing support I have received from my friends and colleagues on the Victoria College Don Team. I have had the pleasure to work with these remarkable individuals and their continual support and encouragement in everything I do, to my Master’s and beyond, mean the world to me.

Each of my mentors has left an impact on my life and to them I am forever indebted, but I would like to take this opportunity to thank those who I have mentored. To my students, who showed me the meaning of strength, persistence, curiosity, and faith, I will cherish all our memories and will forever be grateful for the many lessons you have taught me as well as the strength you have showed me.

Both demanding yet patient, Andrew Woolley has been among the most influential individuals in my life over the past year, serving as a role model for patience, hard work, resilience, and kindness. I could not have asked for a more supportive individual to help guide me on this journey and to him I will forever be thankful.

Great individuals are hard to come by, and I consider myself incredibly lucky to have earned the friendship of so many great individuals. To my friends I am ultimately the most thankful as I would be nothing and nowhere without their continual support and love. Of special mention are Sabina Freiman, Yannay Khaikin, Ashkan Salehi, and Jordan LoMonaco – thank you for continuously renewing my faith in myself.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG</td>
<td>benzylguanine</td>
</tr>
<tr>
<td>Bis-Q</td>
<td>3,3'-bis[α-(trimethylammonium)methyl]azobenzene dibromide</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>¹³C</td>
<td>carbon-13</td>
</tr>
<tr>
<td>Calc.</td>
<td>calculated</td>
</tr>
<tr>
<td>CAPSO</td>
<td>3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid</td>
</tr>
<tr>
<td>cat</td>
<td>catalytic</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>D</td>
<td>deuterium, ²H</td>
</tr>
<tr>
<td>DART</td>
<td>direct analysis in real time mass spectrometry</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOM</td>
<td>dioxane-ortho-methoxy substituted azobenzene</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl, CH₃CH₂</td>
</tr>
<tr>
<td>FDA</td>
<td>(United States) Food and Drug Administration</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein coupled inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$^1$H</td>
<td>proton</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human Embryonic Kidney 293 cells</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>J</td>
<td>joule</td>
</tr>
<tr>
<td>$J$</td>
<td>coupling constant (in spectroscopy)</td>
</tr>
<tr>
<td>K</td>
<td>degree Kelvin</td>
</tr>
<tr>
<td>k</td>
<td>rate constant</td>
</tr>
<tr>
<td>$K_a$</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>wavelength</td>
</tr>
<tr>
<td>LED</td>
<td>light emitting diode</td>
</tr>
<tr>
<td>LOGO</td>
<td>light-operated-GIRK channel opener</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MAGA$_{2p}$</td>
<td>maleimide-azobenzene-glutamate-antenna activated through two-photon (2p) absorption</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass-to-charge ratio (in mass spectrometry)</td>
</tr>
<tr>
<td>Me</td>
<td>methyl, CH$_3$-</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mGlu</td>
<td>metabotropic glutamate</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrum, mass spectrometry</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl, C₆H₅-</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>quadrupole time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>QA</td>
<td>quaternary ammonium</td>
</tr>
<tr>
<td>R</td>
<td>alkyl</td>
</tr>
<tr>
<td>τ₁/₂</td>
<td>half-life</td>
</tr>
<tr>
<td>TD</td>
<td>tetra dioxane substituted azobenzene</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran, C₄H₄O</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TOM</td>
<td>tetra-ortho-methoxy substituted azobenzene</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>visible</td>
</tr>
</tbody>
</table>
Chapter 1
Photocontrol of Biological Systems

1.1 Introduction

As your eyes scan this document, millions of 11-cis-retinal molecules are being photoisomerized (isomerization via photon absorption) to allow you to distinguish black from white forming a picture in your mind’s eye.\textsuperscript{1} This everyday example illustrates the power of photoisomerization, as all of human history—religion, art, and war, has relied upon our ability to visualize our surroundings. Despite the seeming ubiquity of photoisomerization, it was not until the early 1970s that photoswitches were used to control biological functions other than vision, a process termed photopharmacology (see Figure 1).\textsuperscript{2} While using light to control biological phenomena comes with a myriad of benefits, it also bears significant problems that must be addressed before the advent of FDA-approved photopharmaceuticals.

\textbf{Figure 1.} Structure of Bis-Q (a compound developed by Erlanger and colleagues) in its \textit{trans} and \textit{cis} forms. The \textit{trans} form acts as an acetylcholine receptor agonist, while the \textit{cis} form is devoid of activity towards the receptor.\textsuperscript{2}

The most immediate benefit in using light to control biology is the ability to impart precise spatial and temporal control. By virtue of controlling the location and timing of light exposure, biological effects can be controlled in a highly reliable manner.\textsuperscript{3,4,5} This increases our ability to impart selectivity for a particular site, potentially mitigating the side effects present in current pharmaceutical agents. As side effects are mostly due to unwanted activity of a pharmacological agent, this can be avoided simply by controlling when and where the light is shone.\textsuperscript{6} Furthermore, while selective control within the body can minimize side effects of the photopharmaceutical, control outside the body can be important to minimizing drug resistance.
and environmental toxicity.\textsuperscript{7} As multiple strains of multi-drug resistant bacteria arise, the creation of antibiotics that can only be “turned on” when necessary could prove to be of great importance.

An equally important benefit to using light as a stimulus is light’s bioorthogonality towards most biological and chemical systems.\textsuperscript{8} Due to its inability to interact with a majority of biological systems, with the obvious exception of the visual system, light is typically benign and inert. Therefore, light has the unique ability to act as an external stimulus that requires no surgical manipulation and, unlike chemical and biological agents, light cannot cause contamination of a sample. Furthermore, light can be controlled accurately and quantitatively in terms of its intensity and wavelength.

While photopharmaceutical agents promise to overcome the above-mentioned hurdles that conventional drugs face, they are not without drawbacks. Introduction of a photoactive moiety into a drug is far from easily accomplished as drug structures are often highly optimized with even minor changes likely to affect activity. Ideally this moiety would undergo a major change in conformation, polarity, flexibility, and/or electronic effects that would cause two different states to arise in the presence of light and dark.\textsuperscript{9} To maximize a difference in potencies between the light and dark states, a significant change in the aforementioned properties is usually required. Optimization of traditional pharmaceuticals is laborious, and the addition of a photoactive moiety would likely further complicate the process.

Additionally, features of the absorption spectra of the light and dark states often limit the utility of the photopharmaceutical agent. Ideally, the thermodynamically stable form should absorb in the near-infrared window (wavelengths in the range of 700 – 900 nm, see Figure 2).\textsuperscript{10} This allows for minimum scattering and absorption of light by tissue, by hemoglobin on the low end of the range and water at the high end, allowing for the deepest penetration \textit{in vivo}. Furthermore, the ON state should have an absorption profile different from the OFF state. With overlapping absorption spectra, the possibility of simultaneously irradiating both the ON and OFF states arises, creating a photostationary state. This creates an undesirable equilibrium between the states. Ideally one state is active while the other is inactive, but at a photostationary state a population of both active and inactive agent will persist. Notwithstanding this inherent
equilibrium and provided the ON and OFF species have vastly different potencies, an equilibrium would still allow for the therapeutic effects of one state to dominate.

Figure 2. Absorption spectrum from 400 to 2000 nm for human skin measured ex vivo (N = 21 samples).\textsuperscript{11}

Moreover, the half-life of the active form of the photopharmaceutical must be on a suitable timescale for biological utility. The benefits of a photoswitchable pharmaceutical agent would be meaningless if the agent was trapped in one state. While the optimal half-life will vary depending on the use of the photopharmaceutical (e.g. chemotherapeutic vs. antibiotic), a half-life on the order of seconds proves promising for most biological applications, allowing a portion of the agent to bind to its selected target while the remainder of the agent diffuses from the desired site in its inactive form.

It is with these potential strengths and weaknesses that many have turned to azobenzenes. Azobenzenes are a class of photoswitches that have been studied for almost two hundred years, with their potential as photoswitchable mediators of biological control being realized as of the early 1970s.\textsuperscript{2} Upon irradiation, azobenzenes isomerize from the thermodynamically favored \textit{trans} configuration to the \textit{cis} configuration. This presents one of the largest known changes in molecular conformation, a feature that has been exploited in bioactive molecules to present two states; an ON and OFF state. In addition, many of the aforementioned properties of azobenzenes (absorption spectrum, half-life of the \textit{cis} isomer, etc.) as well as many other properties, such as solubility and susceptibility to hydrolysis, are tunable depending on substituents around the phenyl rings of the azobenzene.\textsuperscript{12,13} Due to their relative ease of synthesis as well as their tunable properties and efficient trans-to-cis photoisomerization, azobenzenes have become a powerful
tool in photopharmacology, allowing for the control of numerous biological systems from gene expression to cell signaling.\textsuperscript{14, 15, 16, 17}

1.2 Biological Applications of Azobenzenes

The first use of azobenzenes to impart control over a biological system was in 1971, when Bartels et al. were able to control the firing of electrical potentials in \textit{Electrophorus electricus} (the electric eel).\textsuperscript{2} The photoswitch, termed Bis-Q (see Figure 1), featured a trimethylammonium ligand covalently linked to an azobenzene core. By controlling the \textit{cis}-to-\textit{trans} photoisomerization, control over binding to nicotinic acetylcholine receptors and thus control over the electrogenic membrane of \textit{E. electricus} was accomplished. In the following decade, experiments on photocontrol of muscarinic receptors in frog heart using similar Bis-Q compounds were found to successfully induce conductance.\textsuperscript{2} However, perhaps the richest examples of photocontrol arose when neuroscientists became aware of the utility azobenzene-based compounds can bestow by enabling photo-agonism or photo-anagonism in neuronal circuitry. In their responsiveness to light, an external stimulus enabling accurate spatiotemporal control, azobenzene regulators have allowed advances within neuroscience.

In 2008, Kramer and colleagues synthesized Acrylamide-Azobenzene-Quaternary ammonium (AAQ, see Figure 3).\textsuperscript{18} In cell culture, AAQ was found to cross the cell membrane where the \textit{trans} form of AAQ is able to inhibit endogenous voltage-gated K\textsuperscript{+} channels via quaternary ammonium binding to the intracellular vestibule.\textsuperscript{19} Isomerization using near-UV light (380nm) yielded a significant change in the shape of AAQ, preventing the \textit{cis} form from binding to the channel protein. Voltage clamp recordings showed a significant reduction in membrane current, supporting AAQ’s role as a photoswitchable inhibitor of endogenous voltage-gated K\textsuperscript{+} channel proteins.

Further work on AAQ lead to the synthesis of a similar compound termed QAQ (with a second quaternary ammonium substituted for the acrylamide, see Figure 3). Work on QAQ has focused on nociceptors, with Mouret et al. selectively delivering QAQ to nociceptors, allowing for photo-induced inhibition of nociception (pain) or reactivation by exposure to near-UV light.\textsuperscript{20} The advantages from the AAQ and QAQ photoswitches are numerous; pain inhibition, restoration of
sight, and numerous other biological processes initiated or mediated by action potentials can theoretically be targeted.\textsuperscript{21, 22}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Top: Structure of \textit{trans} (left) and \textit{cis} (right) AAQ. Bottom: Structure of \textit{trans} (left) and \textit{cis} (right) QAQ. Note the triethylammonium moieties highlighted in yellow. Figure adapted from reference 14.\textsuperscript{14}}
\end{figure}

In addition to nociception, other studies have focused on metabotropic glutamate (mGlu) receptor and NMDA receptor photocontrol.\textsuperscript{23, 24} However, these methods rely on genetic modification to ensure tethering of the azobenzene photoswitch to the respective receptor. In 2015, Trauner and colleagues utilized a genetically encoded SNAP-tag in mGlu receptors to selectively bind an azobenzene connected through a benzylguanine \textit{via} a flexible linker (see Figure 4). In HEK-293 cells, patch clamp traces showed reversible inhibition of the modified SNAP-mGlu receptor with photoactivation of the receptor achieved upon illumination with near-UV light (380 nm) and deactivation with green (500 nm) light.
Paralleling Trauner and colleagues’ work with the SNAP-mGlu receptor, Isacoff and colleagues created a set of genetically engineered GluN subunits to which an azobenzene species was attached via a reactive maleimide tag. On the opposite end of this azobenzene lay a glutamate moiety and upon excitation with near-UV light, the resting trans form of the azobenzene isomerized into the active cis form, allowing the glutamate moiety to bind to the receptor creating a photo-agonistic effect. However, while switches such as those presented by Trauner and Iscaoff allow for selective control over specific receptors compared to diffusible agents such as QAQ, the need for genetic modification on endogenous receptors poses a significant drawback for utility in humans.

In 2017, yet another diffusible, azobenzene-based photoswitch was reported by Trauner and colleagues. A novel light-operated-GIRK channel opener (LOGO, see Figure 5) was synthesized and shown to bind selectively to G-protein coupled inwardly rectifying potassium channels (GIRKs). GIRKs are part of inhibitory signal transduction pathways and are ubiquitously expressed throughout the human body. Upon opening, GIRK channels reduce the activity of excitable cells via hyperpolarization, making the channels important in cardiac function, movement coordination, and cognition. LOGO, a diffusible azobenzene, activates GIRK channels in the trans state, but exposure to UV irradiation, and subsequent photoisomerization into the cis state, rapidly silences action potentials. Photoisomerization to silence action

Figure 4. Scheme showing the SNAP-tagged mGlu receptor reacting with the benzylguanine tagged azobenzene. The trans form is inactive and unbound, while the cis form is bound to the receptor and active. Note the long tether allowing for free movement of the azobenzene. Figure adapted from reference 23.
potentials was not only achieved in dissected hippocampal neurons, but also \textit{in vivo} to control the motility of zebrafish larvae.

![Figure 5. Structure of the \textit{trans} LOGO, a known selective GIRK channel blocker.]({})

1.3 Red-Shifted Azobenzene Photoswitches

While a wide array of biological processes have been controlled using azobenzenes, the majority of these examples rely on near-UV and/or blue light as the external stimulus. Light in this region of the spectrum has been shown to induce toxicity in cell culture and damage cells, limiting \textit{in vivo} utility. Biological application is further limited by blue light’s limited penetration of skin (see Figure 2). Therefore, it is clear that a significant red shift in the absorption spectrum of azobenzenes must occur before azobenzenes can be used more extensively in biology.

The effect of varying substitution patterns on the phenyl rings of azobenzenes has been well studied. Addition of electron donating groups at either the \textit{ortho} or \textit{para} position of the phenyl ring results in a bathochromic shift in the absorption spectrum. In addition, incorporation of a push-pull pattern across the \textit{azo} bond, where one ring is electron-rich and the other electron-poor results in further red shifts. However, push-pull azobenzenes have the drawback of having very rapid thermal relaxations limiting their utility in most photoswitching applications. Woolley and colleagues have shown that adjustment of electron donating ability at the \textit{para} position greatly affects the half-life of the \textit{cis} isomer, with an \textit{sp}³ carbon analog having a half-life of 43 hr and an electron rich urea analog having a half-life of 11 sec (see Figure 6 and Table 1).
Figure 6. Structures of select azobenzene photoswitches used for photocontrol. Note that the spectral and physical properties are highly dependent on substitution pattern.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \lambda_{\text{max}} ) (trans)</th>
<th>( \tau_{1/2} ) (cis-to-trans)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>342</td>
<td>43 hr</td>
</tr>
<tr>
<td>2</td>
<td>366</td>
<td>12 min</td>
</tr>
<tr>
<td>3</td>
<td>372</td>
<td>80 sec</td>
</tr>
<tr>
<td>4</td>
<td>382</td>
<td>11 sec</td>
</tr>
<tr>
<td>5</td>
<td>480</td>
<td>25 ms</td>
</tr>
<tr>
<td>6</td>
<td>450</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>10</td>
<td>530</td>
<td>0.7 sec</td>
</tr>
</tbody>
</table>

Table 1. Maximum absorbance and half-life of the cis isomer of select azobenzene photoswitches (structures shown in Figure 6). As a trend, the half-life of the cis isomer shortens as the maximum absorbance of the trans isomer red shifts. Table adapted from reference 12.

One class of azobenzenes has proven successful by possessing both an optimal half-life of the cis isomer and an absorption spectra extending into the near-infrared. BF\(_2\)-coordinated azobenzenes have been shown to be capable of photoisomerizing at wavelengths within the near IR window and their cis isomers have half-lives between 4 and 15 min (see Figure 7.). The major drawback to these BF\(_2\) adducts is their instability in aqueous solvent. Photoisomerization was conducted in dichloromethane as the BF\(_2\)-coordinated azobenzenes hydrolyze into their respective hydrazone with half-lives of hydrolysis ranging from 1 to 2.8 hours.\(^{32}\)
BF₂-coordinated azobenzene designed by Aprahamian and colleagues. This and other BF₂ derivatives hydrolyze slowly in aqueous solutions to their corresponding hydrazones.

An alternative solution to circumventing the limited penetration of blue light into tissue is to utilize two-photon absorptions. However, this strategy is not without its own difficulties as it would require a high intensity of light to sufficiently isomerize the azobenzene. Furthermore, the structure of azobenzenes absorbing two-photons necessitates the incorporation of large, conjugated groups to serve as “harvesting antennae” for photon absorption (see Figure 8). These bulking antennae complicate synthesis and may impair the ability of the azobenzene to exhibit different bioactivities in its cis and trans states.

Figure 8. Structure of MAGA₂p (maleimide-azobenzene-glutamate-antenna) activated through two-photon (2p) absorption. The maleimide moiety (green) allows for covalent linkage to the glutamate receptor (GluR), while the azobenzene moiety (red) provides changes in conformation freeing or concealing the glutamate agonist (blue). The naphthalene moiety (pink) represents the harvesting antenna for photon absorption.

To date, the most successful red-shifts that offer longer half-lives for the cis isomer involve tetra ortho substitution patterns (see Figure 9). Examples of tetra ortho chloro, bromo, methoxy, and ethyl thioether substituents along the phenyl rings of the azobenzene also have the added benefit of showing significant separation between the absorption spectra of the trans and cis isomers, making it easier to create a larger proportion of one isomer at a given time.
Furthermore, the presence of para amines has been observed to affect the absorption spectrum of azobenzenes resulting in even greater red-shifts. By changing the amine at the para position, the degree of delocalization of the amine lone pairs into the phenyl rings can be altered allowing for significant red-shifts. In addition to a bathochromic shift, the presence of para amines also plays a part in raising the pK_a of the corresponding azonium ions (see Figure 10). Woolley and colleagues observed that ortho methoxy substitution of phenyl rings allows for the stabilization of the azonium ion while simultaneously serving as electron donating groups. When used in combination with the para amino substitution, this tetra ortho methoxy (TOM) substitution pattern yields a significantly red-shifted azobenzene with a pK_a of the corresponding azonium within the physiological range. Further red-shifts were observed when meta electron donating groups (eg. methoxy substituents) were added onto the phenyl ring.
Figure 10. Previous \( p \)-amino TOM azobenzenes in their protonated (azonium) states.\(^{38} \) The identity of the \( p \)-amino substituent plays a large role in the pK\(_a\) of the azonium as can be seen in the difference in acidity between compounds 15 and 16, which differ only in their \( para \) substituent.

1.4 Motivation and Direction

Our group has found success in using tetra \textit{ortho} methoxy (TOM) substituted azobenzenes as red-light switchable groups that can be used to control peptide conformation. Using this azobenzene motif, control over the helix-to-coil transitions in FK-11 and a fluorescent analog (Fl-(Pro)\(_9\)-FK11) were achieved.\(^{31} \) Previous work with TOM azobenzenes, which are among the first red-shifted azobenzenes to be used in biological applications, serves as the foundation for the work described herein. With their predictable \textit{trans-to-cis} isomerization, stability in aqueous media, and red-shifted absorption spectra, TOM azobenzenes provide a useful starting point for further modification and improvement in the search for physiologically suitable photoswitches.

1.5 References


Chapter 2
Towards Optimal Red-Shifted Azobenzenes

2.1 Research Goals

Our goal was to synthesize a novel azobenzene that had favorable properties for use as a photoswitch in biological applications. As described above, the TOM substitution pattern was shown to offer a significant red shift in the absorption spectrum and, depending on the para-substituted amine, showed promising thermal relaxation rates. The TOM motif and the presence of para amines also served to increase the pK$_a$ of the corresponding azonium ions, resulting in azobenzenes that are not only bathochromically shifted, but also form a significant population of azonium ions at physiological pH. Previous work (described above) showed that substitution of electron donating groups at the meta position, in addition to the ortho and para positions, would lead to an even more significant red shift in the absorption spectrum.\textsuperscript{1} To prevent steric clashes between neighboring methoxy groups, which would negate the conjugation between the oxygen lone pairs and the aromatic system, we had originally planned to have such groups tethered, resulting in four, fused dioxane substituents spanning both phenyl rings of the azobenzene core (dubbed the TD, or tetra dioxane, pattern). However, due to technical issues such as solubility of precursors, an azobenzene with a TD substitution pattern was unable to be synthesized. As a compromise between solubility and bathochromic shifting, only two fused dioxane rings and two methoxy substituents were chosen (dubbed the DOM substitution pattern, see Figure 11).

![Figure 11](image)

**Figure 11.** From left to right, the tetra ortho methoxy (TOM), tetra dioxane (TD), and dioxane ortho methoxy (DOM) azobenzene motifs.
To study the properties of the DOM azobenzene core, it was coupled to a pyrrolidine moiety (17) and to an azetidine (18). This provided two azobenzenes with electron donating groups at the ortho, meta, and para positions of the phenyl rings. As aggregation in solution was observed with similar compounds, we also included groups designed to enhance water solubility. The inclusion of the methyl sulfone moiety was intended to increase water solubility of 17. Dimethyl amino groups (which should be protonated at physiological pHs) were included to increase the water solubility of 18. The distance of these functional groups from the azobenzene core was such that we expected little to no effect on the spectral properties of the azobenzene core.

![Structures of two lead azobenzenes, both exhibiting a DOM substitution pattern and para electron donating groups.](image)

**Figure 12.** Structures of two lead azobenzenes, both exhibiting a DOM substitution pattern and para electron donating groups.

### 2.2 Synthesis of 17 and 18

**General**

All commercial materials (solvents and reagents) were used as received unless otherwise indicated. SiliaFlash P60 (Silicycle Inc.) silica gel of particle size 40-63 μm was used for column chromatography. High performance liquid chromatography was run on a PerkinElmer Series 200 pump with a Waters 2487 Dual λ Absorbance detector connected to an eDAQ PowerChrom 280 recorder. One-dimensional $^1$H and $^{13}$C NMR as well as two-dimensional gCOSY and HSQC spectra were recorded on Varian UnityPlus 500 MHz, Varian Mercury 400 MHz or Agilent 700 MHz spectrometers. The chemical shifts of the spectra are reported in parts per million (ppm) and the spectra were referenced to the residual NMR solvent signals. For $^1$H NMR: chloroform-d (7.26 ppm), methanol-d4 (3.31 ppm), and DMSO-d6 (2.50 ppm). For $^{13}$C NMR: chloroform-d (77.16 ppm), methanol-d4 (49.00 ppm), and DMSO-d6 (39.52 ppm). $^1$H NMR spectral characterization is reported as follows: chemical shift (multiplicity, coupling constant, integration, assignment). Mass spectra were recorded using an Agilent 6538 mass spectrometer.
with a Q-TOF ionization source or a JEOL AccuTOF mass spectrometer with a DART ionization source.

\((E)-1,2\text{-bis}(6\text{-methoxy}-8\text{-}((S)\text{-3-(methylsulfonyl)pyrrolidin-1-y})\text{-2,3 dihydrobenzo}[\text{b}]\text{[1,4]dioxin-5-yl]diazene (17)}\)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 1,2-bis(8-bromo-6-methoxy-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)diazene (obtained from Prof. Mingxin Dong, Beijing Institute of Biotechnology) (203 mg, 0.39 mmol), (S)-3-(methylsulfonyl)pyrrolidine (AstaTech Inc.) (430 mg, 2.88 mmol), RuPhos (3.2 mg, cat.), Pd\(_2\)(dba)_3 (3.2 mg, cat.), Cs\(_2\)CO\(_3\) (1.15 g, 3.53 mmol) and anhydrous toluene (3 mL). The tube was sealed with a Teflon stopper and heated to 100°C for 36 h. The reaction was cooled to room temperature and the solvent was removed in vacuo. The crude material was dissolved in EtOAc, washed with brine, dried with Na\(_2\)SO\(_4\), and concentrated in vacuo. Column chromatography (methanol: DCM (1:9)) yielded the semi-pure product as a blue solid. Reverse-phase HPLC (Zorbax RX-C18 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid (TFA) over 22 min) yielded the pure product as a blue solid. As the product came off the HPLC, saturated sodium bicarbonate solution was added to neutralize the TFA. \(^1\)H NMR (500 MHz, chloroform-\(d\)) \(\delta\) 6.03 (s, 2H, H\(_a\)), 4.38-4.33 (m, 4H, H\(_b\)), 4.32-4.26 (m, 4H, H\(_c\)), 4.00-3.94 (m, 2H, H\(_g\)), 3.83 (s, 6H, H\(_e\)), 3.75-3.69 (m, 4H, H\(_d\)), 3.56-3.50 (m, 2H, H\(_i\)), 3.50-3.44 (m, 2H, H\(_d\)), 2.95 (s, 6H, H\(_j\)), 2.43 (m, 4H, H\(_h\)) ppm. \(^{13}\)C NMR (125 MHz, chloroform-\(d\)) \(\delta\) 147.96 (C6), 138.93 (C4), 138.19 (C1 and C2), 129.24 (C3) 94.42 (C5), 64.89 (C7), 63.58 (C8), 61.74 (C13), 57.41 (C9), 50.28 (C11), 49.54 (C10), 38.79 (C14), 25.68 (C12) ppm. ESI-Q-TOF: calculated m/z for C\(_{28}\)H\(_{36}\)N\(_4\)O\(_{10}\)S\(_2\): 653.1959 [M+H\(^{+}\)], found 653.1954.

The structure of 17 was confirmed by mass spectroscopy as well as NMR characterization. Assignment of the H nuclei was confounded by the presence of a stereocenter at C13 (the 3 position of the pyrrolidine ring). This creates diastereomeric H nuclei throughout the pyrrolidine ring. Through HSQC and gCOSY NMR, the diastereomeric H nuclei were identified and assigned. Another difficulty arose in the \(^{13}\)CNMR assignments, particularly the C nuclei lacking an H atom (preventing characterization through HSQC) as well as C nuclei that are adjacent and
have similar numbers of H atoms (i.e. the dioxane C nuclei). For these assignments, ChemDraw predication software was used and the most downfield C nuclei assigned via the software, were assigned the most downfield C nuclei in the $^{13}$CNMR

(E)-1,2-bis(6-methoxy-8-(2-((dimethylamino)methyl)azetidin-1-yl)-2,3 dihydrobenzo[b][1,4]dioxin-5-yl)diazene (18)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 1,2-bis(8-bromo-6-methoxy-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)diazene (70 mg, 0.14 mmol), azetidin-3-yl-N,N-dimethylmethanamine·HCl (Enamine) (70mg, 0.46 mmol), RuPhos (8.5 mg, cat.), Pd2(dba)3 (9.8 mg, cat.), Cs2CO3 (480 mg, 0.30 mmol) and anhydrous toluene (4.0 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 36 h. The crude product was diluted with ethyl acetate, washed with saturated sodium bicarbonate and concentrated in vacuo. The crude product was subjected to column chromatography (methanol: DCM (1:19)) yielding the semi-pure product as a blue solid. Reverse-phase HPLC (Zorbax RX-C18 column with a linear gradient from 5% to 70% ACN/water containing 0.1% TFA over 25 min) yielded the pure product as a blue solid (yield % by HPLC). $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 2.24 (s, 12H, Ha), 2.58 (d, 4H, He,), 2.85 (septet, 2H, Hd), 3.69 (dd, 4H, Hb), 3.82 (s, 6H, Hf), 4.25-4.13 (m, 8H, Hg), 4.31-4.35 (m, 4H, Hc,), 5.62 (s, 2H, Hh) ppm. $^{13}$C NMR (125 MHz, Chloroform-d) $\delta$ 29.25 (C3), 45.66 (C1), 56.81 (C12), 58.16 (C4), 63.10 (C11), 63.63 (C2), 64.99 (C8), 89.88 (C6), 127.08 (C10), 138.69 (C9), 143.32 (C7), 149.12 (C5) ppm. ESI-Q-TOF: m/z calc. for C28H36N4O8: 557.2606 [M+H]+; found 557.2596

Figure 13. Synthetic scheme for the synthesis of 17 and 18 via a Buchwald-Hartwig type reaction. Note both 17 and 18 are tertiary aryl amines.
2.3 UV-Vis absorption spectra of 17 and 18

Following the synthesis of compounds 17 and 18, we were interested in exploring their spectral properties. To serve as optimal photoswitches, the azobenzenes had to exhibit absorption bands that extended into the near infrared region of the electromagnetic spectrum.

Solutions were prepared with the compounds dissolved in 20% DMSO in 5 mM of Universal Buffer (CAPSO, TRIS, MES, sodium acetate, 5 mM each) to ensure the pH could be adjusted between 3 and 11 by addition of small quantities hydrochloric acid (2 M) or sodium hydroxide (2 M). An aliquot was placed in a quartz cuvette with a light path length of 10 mm. Small quantities of aqueous hydrochloric acid or sodium hydroxide were added directly to the cuvette and the resulting pH was measured by a glass combination micro-electrode (MI-710, Microelectrodes Inc.) UV-Vis spectra were acquired using a PerkinElmer Lambda-35 or Lambda-25 instrument coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc.). The temperature was maintained at 20°C throughout the experiments. All spectra were baseline-corrected, assuming zero absorption at 850 nm.

Upon measuring the absorption spectra of 17, a few key findings were made (see Figure 15). Most easily identified is the pH-dependent nature of the absorption spectrum. A basic pH favours formation of the neutral azobenzene, which absorbs maximally at 482 nm. With increasing acidity, the protonated azonium ion arises which has a significant bathochromic shift in its absorption spectrum compared to the neutral species, with an absorption maximum at 596 nm. Furthermore, the isosbestic point of the two forms of the azobenzene was determined to be 501 nm. Of most interest is the broad absorption band of the azonium form of 17, where a significant portion of the tail falls in the near-infrared region (i.e. >700 nm) In principle, this allows for the use of near-IR light to induce isomerization. Of equal importance is the pKₐ of the compound, which was determined to be 6.7. As it is the protonated form that absorbs in the near-IR region, formation of the azonium ion must be favoured at physiological pH for this azobenzene motif to be present within biological systems. Thus, higher pKₐ are favoured, but even at a physiological pH of 7.2, the azonium form is still present allowing a portion of the compound to absorb near-IR light at physiological pH.

Compound 17 can be compared to a similar TOM derivative (19), containing the TOM motif in place of DOM, and a 3-methoxypyrrolidine in place of a 3-sulfonpyrrolidine (see Figure 14).²
While 19 had a much more favourable pKₐ of 9.1, the absorption maxima for the azonium was only 560 nm with the absorption band quickly dropping off past 680 nm (see Figure 15).

Figure 14. Structures of 17 and 19 for comparison. Note that while 17 contains a DOM motif, 19 contains a TOM motif. Both contain 3-substituted pyrrolidine rings at the *para* positions.

Figure 15. pH titration of 17 (left) and 19 (right). Note the formation of the red-shifted azonium species at lower pH. 17 shows a pKₐ of 6.7 and an absorption maximum at 596 nm, while 19 shows a pKₐ of 9.1 and an absorption maximum at 560 nm.

The UV-Vis spectra of compound 18, are shown in Figure 17. The neutral azobenzene absorbs maximally at 486 nm, while the azonium ion of 18 has an absorption maximum at 598 nm (see Figure 16). Furthermore, the isosbestic point of the two forms of the azobenzene was determined to be 500 nm. The pKₐ of the compound, determined to be 6.4, is lower than that of 17 (6.7). A similar TOM analog, bearing a 3-methoxy azetidine (in place of a 3-methylenedimethylamine azetidine) was also previously synthesized (20, see Figure 16).¹ In comparison, 20 has a much higher pKₐ (8.3), but a significantly less red-shifted absorption maximum (545 nm).
Figure 16. Structures of 18 and 20 for comparison. Note that while 18 contains a DOM motif, 20 contains a TOM motif. Both contain 2-sustituted azetidine rings at the para positions.

Figure 17. pH titration of 18 (left) and 20 (right). Note the formation of the red-shifted azonium species at lower pH. 18 shows a pK\(_a\) of 6.4 and an absorption maximum at 598 nm, while 20 shows a pK\(_a\) of 8.3 and an absorption maximum at 545 nm.

The absorption maxima for the 17 and 18 were almost identical, with a maximum absorption of 486 and 482 nm (respectively) for the neutral species, and 595 and 598 nm (respectively), for the azonium forms. Similarly, solubility was not seen to be an issue for either compound in 20% DMSO aqueous solutions on a short timescale. However, when left for extended periods (days to weeks) in 20% DMSO, aggregates began to form in solution. This aggregation was observed to worsen near the pK\(_a\) of the compounds and particularly at lower pH. When methanol is substituted for DMSO, blue aggregates are observed to form much quicker (within minutes to hours).

When proceeding with photoisomerization trials, 17 was chosen over 18 for two reasons (see Table 2 for comparisons). Firstly, the pK\(_a\) of 18 (6.4) was determined to be slightly lower than that of 17 (6.7). This means that a smaller proportion of the protonated azonium form of 18 will be present at physiological pH and, as it is the protonated azonium that is strongly red-shifted, a
large population of azonium is required. Secondly, 17 has a wider absorption band compared to 18, extending further into the near-infrared range. Thus, while their absorption maxima are very similar, the wider absorption band of 17 made it the more suitable option for photoisomerization.

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<th>19</th>
<th>20</th>
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<td>DOM</td>
<td>TOM</td>
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</tr>
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<td>598 nm</td>
<td>560 nm</td>
<td>545 nm</td>
</tr>
</tbody>
</table>

Table 2. Comparisons of properties determined via pH titration of various DOM and TOM azobenzenes.

2.4 Photoisomerization of 17

With the success of synthesizing an azobenzene with an absorption profile extending into the near-infrared region of the spectrum, we were interested in the photoisomerization properties of 17.

A solution of 17 was prepared in the Universal Buffer Mixture described in section 2.3. For each measurement, a quartz cuvette with a light path length of 10 mm was used. A green LED (LedEngin LZ4-41G100, 700 mA, 537 nm, 330 mW/in$^2$) was used as a source for the measuring beam while isomerization was achieved by irradiating with two near infrared (Thor Labs, 720 nm, 8.61V, 0.477A, 55mW/in$^2$ each) high power LEDs. Thermal relaxation rates were measured by monitoring absorbance after removal of the near infrared light source. Relative absorbance values were recorded using a photomultiplier tube (Oriel, Newport Corporation, set to 360V) connected to a digital oscilloscope (Handyscope HS3, TiePie Engineering). One linear variable band-pass filter (LVF HL, Ocean Optics) transmitting at 540 ± 20 nm was placed before and in front of the sample to eliminate other wavelengths, including scattered light coming from the isomerization light source. A second band-pass filter transmitting at 540 ± 20 nm (LVF-HL, Ocean Optics) was placed in front of the detector to further block the scattered light from reaching the detector. The output of the detector was recorded immediately after triggering the red light off. Fitting the photomultiplier tube signal vs. elapsed time to a monoexponential decay process gave the observed kinetic
constants for thermal relaxation. The temperature of the sample was maintained at 20 °C during all measurements using a temperature controlled cuvette holder (Quantum Northwest, Inc.).

Photoisomerization was accomplished under near-physiological conditions (aqueous buffer solution containing 20% DMSO, pH 7.2 at 20°C). Through irradiation with 720 nm light, a change in absorbance intensity is seen, corresponding to a change in the concentration of trans 17 (see Figure 18). The half life of the cis isomer was observed to be 0.8 seconds.

![Figure 18. Photoswitching of 17 with near-infrared light at physiological conditions (20% DMSO in 5 mM Universal Buffer at pH 7.2).](image)
Figure 19. Relaxation of 17 after irradiation with near-infrared light at physiological conditions (20% DMSO in 5 mM Universal Buffer at pH 7.2). A monoexponential fit was applied, showing a half-life of 1.0 seconds.

2.5 Molar Absorptivity of 17

Ideally, a photoswitch will have a high molar extinction coefficient (ε) allowing it to efficiently absorb light. Therefore, after proving compound 17 was capable of undergoing photoisomerization and had a favorable relaxation rate, we set out to determine the molar extinction coefficient of 17. To calculate the molar extinction coefficient, we utilized p-nitrophenol (see Figure 20) as an internal standard with a known molar absorption coefficient.3

Figure 20. p-nitrophenol and its corresponding phenolate, which has a ε = 18465 M⁻¹ cm⁻¹ at 400 nm
A 17 stock solution was created from an indeterminate amount of 17 and 300 µL of DMSO-d₆. Three separate replicates were created (C1, C2, C3), by diluting the 17 stock solution with DMSO-d₆. OD measurements were taken by obtaining a sample from each replicate and diluting 200-1000 fold in universal buffer, containing 20% DMSO. The OD sample for each replicate was basified to between pH 9.7-10.6 and for each replicate, two absorption spectra were obtained and averaged. The OD samples were then acidified to between pH 3.2-4.5, and two absorption spectra were obtained for each replicate. Changing the pH did not substantially chance the volume of the solutions. The maximum absorption for both the azo (481 nm) and azonium (597 nm) species was measured.

A separate p-nitrophenol stock solution was created by dissolving 8.8 mg of p-nitrophenol in 1000 µL of DMSO-d₆. The p-nitrophenol UV-Vis spectrum was obtained by diluting 1000-fold with 20% DMSO in universal buffer. The sample was basified to pH 11.2 and the OD reading (405 nm) was taken. Using the OD from the p-nitrophenol reading, the concentration of p-nitrophenol in the OD sample was determined (using a molar extinction coefficient of 18465 at 405 nm).²

An aliquot (20 µL) of p-nitrophenol stock solution was then added to each replicate and ¹H NMR spectra were obtained for the three replicates. Peak areas (p-nitrophenol peak at 8.12 ppm, 17 at 6.06)) were integrated on IGOR. From their relative peak areas the mole ratio of p-nitrophenol to 17 was obtained. Using the concentration in the NMR tube, the concentration in the 17 stock was determined, and thus the concentration in each OD reading was determined. Finally, knowing the concentration in the OD samples and the OD readings, the molar extinction coefficients were determined. Values were:

Azonium: 138,000 (± 5000) M⁻¹cm⁻¹ at 597 nm

Neutral: 55,000 (± 3000) M⁻¹cm⁻¹ at 481 nm
**Figure 21.** OD vs. concentration graphs for the neutral (left) and azonium (right) forms of 17. The slope represents the molar extinction coefficient (as $l = 1$ cm). $\varepsilon = 5.34 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.35 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for the neutral and azonium forms, respectively.

### 2.6 Glutathione Reduction & pH stability of 17

When exploring the stability of 17, we were interested in the redox stability of the *azo* bond to glutathione. Glutathione is an important reducing agent present within cells at millimolar concentrations, playing the important role of an antioxidant. Studies conducted by Moroder and colleagues show that glutathione is capable of reducing the *azo* bond in solution. Rates of reaction are highly variable, with the fastest reductions occurring in electron deficient azobenzenes. A rapid rate of reduction would preclude use of an azobenzene derivative inside cells, and restrict is use to extracellular, oxidizing environments.

We monitored the stability of 17 over a period of 6 hours in a 10 mM glutathione solution (in degassed 20% DMSO with 5 mM aqueous Universal Buffer solution). An aliquot was placed in a quartz cuvette with a light path length of 10 mm, which was sealed with Parafilm® to prevent exposure to atmospheric oxidizing agents. UV-Vis spectra were acquired using a PerkinElmer Lambda-35 instrument coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc.). The temperature was maintained at 20°C throughout the experiments. A full spectral reading from 850 to 250 nm was performed every 15 minutes for 12 hours. All spectra were baseline-corrected, assuming zero absorption at 850 nm. By fitting the disappearance at 482 nm
to an exponential decay, we observed a slow degradation ($t_{1/2} = 202$ min, see Figure 22). This rate is similar to those previously observed for related azonium ions and may restrict the use of 17 to extracellular environments in vivo.\(^6\)

**Figure 22.** Glutathione reduction assay, showing a half-life of 202 min for 17 in 10 mM glutathione solution.

**Figure 23.** Glutathione reduction assay of 17, measurement of absorbance at 483 nm (absorption maximum of the azobenzene) was plotted over time and fit to a mono exponential decay. From this the rate constant was used to determine the half-life.
2.7 Long term aqueous stability of 17

To further test the stability of 17, we were interested in testing the stability of the compound in aqueous solvent at near-physiological pH. To do this, we examined the absorption spectrum for the compound in aqueous solvent for at least 6 hours at a pH of 6.5, 7, and 7.5 (see Figure 24). The UV-Vis spectra were obtained as described above, with the exception of solvent conditions (10% DMSO in aqueous Universal Buffer was used).

Figure 24. 17 in pH 6, 6.5, 7, and 7.5 for 6-7 hours. The lack of any appreciable change leads us to conclude the compound is reasonably stable within the physiologically relevant pH range of 6-7.5.

The lack of appreciable change over the course of 6 hours leads us to conclude that the compound is stable at these pH, and will not be subject to degradation under these conditions.

Furthermore, to observe stability over longer time periods (i.e. months) in aqueous solution, five solutions of 17 in 20% DMSO in 5 mM Universal Buffer were prepared. The pH of the solutions
was adjusted to 6.5, 7, 7.5, 8.0, and 8.5 and the solutions were left in ambient lighting at room temperature, in sealed tubes for approximately 3 months. The aqueous layers were extracted with DCM following the 3 month period and a TLC was run (see Figure 25).

Figure 25. Thin layer chromatogram of DCM extracts of solutions of 17 that had been stored in universal buffer, 20% DMSO for 3 months (at the pH values indicated). A – pH 6.5; B – pH 7, C – pH 7.5, D – pH 8.0, E – pH 8.5, F – HPLC purified (fresh). Solvent conditions: DCM/MeOH 9/1.

The results of the TLC further suggest that 17 is stable in aqueous solvent for much longer periods, persisting in aqueous solution for months at physiological pH ranges.

2.8 Conclusion

Two compounds, 17 and 18 were synthesized with the DOM azobenzene motif. Both compounds showed strongly red-shifted absorption spectra, with absorption maxima at 596 nm (17) and 598 nm (18). Furthermore, both compounds have a similar propensity for forming the azonium ion in solution as the compounds have similar pKₐ, 6.7 for 17 and 6.4 for 18. Both DOM compounds experienced significant bathochromic shifts compared to their TOM analogs (19 and 20), with an average red shift of 45 nm.

In addition, our work with 17 marks the first observation of photoisomerization using near-IR light under physiological conditions wherein the photoswitch is stable. With a molar extinction
coefficient of $5.34 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ (neutral) and $1.35 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ (azonium), 17 can efficiently absorb light to form the cis isomer in solution. The half-life of the cis isomer was calculated to be 0.8 seconds, along the desired magnitude for biological control.

Furthermore, 17 shows susceptibility to glutathione reduction likely limiting its utility for use in intracellular capacities. In a 10 mM solution of glutathione, 17 was shown to have a half-life of 202 min. The compound also appears to be stable in aqueous solvents across the physiological range from 6 to 7.5, showing little degradation when left in solution for 6 hours or longer.

Compound 17 serves as a model to illustrate the utility of our novel DOM azobenzene core. The DOM substitution pattern allows for a bathochromic shift in the absorption spectrum of azobenzenes, yields optimal molar absorptivity, and is stable to degradation by glutathione and acid/base (within the physiological range).

While 15 proves to be a promising azobenzene for biological use, the compound falls short in utility as it lacks functional handles by which it can covalently bond to biomolecules to exert control over biological systems.

2.9 References


Chapter 3
Introduction of Handles for Conjugation to Red-Shifted Azobenzenes

3.1 Introduction

In Chapter 2, the synthesis and properties of a novel azobenzene capable of absorbing in the near-IR region was discussed. However as mentioned in Chapter 1, the utility in azobenzenes is in their ability to be conjugated to biologically active molecules, such as proteins, nucleic acids, or pharmacological agents. It is through the introduction of the photo-inducible moiety to a bioactive molecule that light-responsive control can be introduced. From conception, compounds 17, 18, 19, and 20 lacked a site of conjugation. Azobenzenes used for biological control require a functional group suitable for conjugation to a bioactive molecule. Examples of such groups are illustrated by the use of threoninol linkers for making azobenzene modified DNA and maleimide and iodoacetamide groups for linking azobenzenes to thiol groups in proteins.\(^1\)\(^2\) Compound 17 exhibited favourable physical properties, including a fairly high pK\(_a\), ideal half-life of the \(cis\) isomer, and an absorption spectrum that extended into the red and near-infrared range. Yet, no further modifications to the compound are easily attainable at the sulfone. Further limiting chemical transformation is the \(azo\) bond’s susceptibility to acidic degradation and reduction. It is with these challenges in mind that we set out to develop variants of 17 containing handles suitable for conjugation.

With the goal of creating 22, we began with \(para\)-diamino form of our azobenzene core. After repeated experiments varying the reaction conditions, addition of the glycine was not accomplished.

![Figure 26](image)

**Figure 26.** Attempted synthesis of 22, which proved to be unsuccessful.
We continued by attempting to couple an ethylenediamine linker in place of a glycine. In theory, the ethylenediamine linker would introduce a functional handle and the presence of a secondary aryl amine would likely provide different photophysical properties in 22 compared to our previous tertiary aryl amines. However, reactions with ethylenediamine consumed the starting material, but showed no appearance of the desired product.

We explored a variety of conditions for coupling a primary amine to our azobenzene after our initial failure to couple to the dibromo compound (26) using the standard conditions used to synthesize 17 and 18 above.\textsuperscript{3,4} Optimization was performed using octylamine, a cheap primary amine with a high boiling point (since the reaction runs at 100°C for over 12 hours). Furthermore, we utilized the TOM azobenzene core (26) as it is more easily produced compared to the DOM analog. By exchanging the ligand and base, we successfully coupled a primary amine to our azobenzene core (see Figure 27).

\textbf{Figure 27.} Attempted coupling of a primary amine to the TOM azobenzene core under a varying conditions. Note only the final set of conditions produced the desired product.
We returned to ethylenediamine to attempt to couple it to our azobenzene core using the modified reaction conditions, but to our surprise the starting material was consumed without appearance of the product. Our assumption was that perhaps the second free amine was somehow interfering with the reaction. To test this assumption, we attempted to couple a diamine with a longer hydrocarbon linker between the free amines, 1,6-diaminohexane using the same, modified reaction conditions. The reaction proceed to completion forming the disubstituted azobenzene, using what we believe to be the first successful coupling of a primary amine at the para positions of an azobenzene with electron donating substituents at both the ortho and meta positions of the rings (see Figure 28).

![Figure 28](image)

**Figure 28.** The successful coupling of a primary amine to an azobenzene core. The resulting secondary aryl amine (27) has different electronic properties from previously synthesized aryl amides and tertiary aryl amines, resulting in different physical properties.

### 3.2 Synthetic Methods

**General**

All commercial materials (solvents and reagents) were used as received unless otherwise indicated. SiliaFlash P60 (Silicycle Inc.) silica gel of particle size 40-63 μm was used for column chromatography. High performance liquid chromatography was run on a PerkinElmer Series 200 pump with a Waters 2487 Dual λ Absorbance detector connected to an eDAQ PowerChrom 280 recorder. One-dimensional $^1$H and $^{13}$C NMR as well as two-dimensional gCOSY and HSQC spectra were recorded on Varian UnityPlus 500 MHz, Varian Mercury 400 MHz or Agilent 700 MHz spectrometers. The chemical shifts of the spectra are reported in parts per million (ppm).
and the spectra were referenced to the residual NMR solvent signals. For $^1$H NMR: chloroform-d (7.26 ppm), methanol-d4 (3.31 ppm), and DMSO-d6 (2.50 ppm). For $^{13}$C NMR: chloroform-d (77.16 ppm), methanol-d4 (49.00 ppm), and DMSO-d6 (39.52 ppm). $^1$H NMR spectral characterization is reported as follows: chemical shift (multiplicity, coupling constant, integration, assignment). Mass spectra were recorded using an Agilent 6538 mass spectrometer with a Q-TOF ionization source or a JEOL AccuTOF mass spectrometer with a DART ionization source.

1,3-dimethoxy-2-nitrobenzene (23)

To a solution of 2-nitroresorcinol (4.01 g, 25.9 mmol) and anhydrous potassium carbonate (10.7 g, 77.4 mmol) in anhydrous DMF (90 mL) was added CH$_3$I (1.9 mL, 75.9 mmol) and the mixture was stirred at room temperature for 5 h. The solution was extracted with ethyl acetate and the combined organic layers were washed with brine, dried over anhydrous Na$_2$SO$_4$ and concentrated under vacuum. The crude product was pure enough for the next reaction (yellow-brown solid, 3.48 g, 73% yield).

1,3-dimethoxyaniline (24)

To a vigorously stirring solution of 21 (3.48 g, 19 mmol) and ammonium chloride (6.91 g, 129 mmol) in water/methanol (1:5), iron filings (3.67 g, 65.7 mmol) were added and the solution was heated to a reflux for 6 h. The reaction was cooled to room temperature, diluted with water then the methanol was removed in vacuo. The aqueous solution was filtered through Celite® and extracted with DCM. The combined organic layers were washed with brine, dried over Na$_2$SO$_4$ and concentrated in vacuo. Column chromatography (ethyl acetate: hexane (1:6)) yielded the pure product as pale pink-white crystals (1.7 g, yield 59%).

1,3-dimethoxy-4-bromo-aniline (25)

A solution of bromine (0.58 mL, 22.8 mmol) in DCM (10 mL) was slowly added to a solution of 22 (1.7 g, 11.1 mmol) in DCM (140 mL) at 0°C over
20 min. The reaction mixture was stirred for 20 min at room temperature and then quenched with NaOH (1 M, 25 mL). The product was extracted with DCM and the combined organics were washed with brine, dried over Na$_2$SO$_4$ and concentrated in vacuo to obtain the crude product. Column chromatography (DCM (100%)) eluted the pure product as light pink crystals (1.3 g, yield 51%).

**1,2-bis(4-bromo-2,6-dimethoxyphenyl)diazene (26)**

To a solution of 23 (1.3 g, 5.2 mmol) in DCM (100 mL) was added KMnO$_4$ (3.2 g) and CuSO$_4$·5H$_2$O (3.1 g) and the reaction was heated to a reflux for 40 h. The crude solution was cooled to room temperature, filtered through Celite® and dried in vacuo. Column chromatography (ethyl acetate: hexanes (1:4)) yielded the pure product as a red solid (305 mg, yield 24%). $^1$H NMR (399 MHz, DMSO-d$_6$) $\delta$ 3.58 (s, 3.2H, OCH$_3$, cis), 3.76 (s, 11.1H, OCH$_3$, trans), 6.79 (s, 1.5H, Ar, cis), 7.01 (s, 4H, Ar, trans). Ratio trans:cis (2.8:1).

**(E)-N$_1^1$N$_1^{11}$-(diazene-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis(hexane-1,6-diamine) (27)**

To an oven-dried pressure tube, cooled under nitrogen gas, was added 24 (30 mg, 0.065 mmol), 1,6-diamonohexane (70 mg, 2.88 mmol), (R)-BINAP (4 mg, cat.), Pd$_2$(dba)$_3$ (6.9 mg, cat.), NaOtBu (103 mg, 3.53 mmol) and anhydrous toluene (3 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 16 h. The reaction was cooled to room temperature and the solvent was removed in vacuo. The crude material was dissolved in DCM, washed with brine, dried with Na$_2$SO$_4$, and concentrated in vacuo. Reverse-phase HPLC (Zorbax RX-C18 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 22 min) yielded the pure product as a blue solid. Note: as the product came off the HPLC, saturated sodium bicarbonate solution was added to neutralize the TFA. $^1$H NMR (500 MHz, chloroform-d) $\delta$ 5.98 (s, 2H, Ar), 4.22(dd, 4H), 3.79 (s, 12H), 1.70-1.40 (m, 14H), 0.95 (m, 6H) ppm. $^{13}$C NMR (125 MHz, chloroform-d) $\delta$ 169.38, 133.55, 132.43, 129.85, 69.13, 40.16, 31.61, 30.13, 24.95, 24.02, 14.40, 11.41 ppm. ESI-Q-TOF: calculated m/z for C$_{28}$H$_{36}$N$_4$O$_{10}$S$_2$: 653.1959 [M+H$^+$], found 653.1954.
3.3 UV-Vis absorption spectra of 27

As 27 possessed a secondary aryl amine at the para position of azobenzene, we were interested in its physical properties, specifically in comparison to pre-existing azobenzenes, none of which contain para secondary amines.

A solution of 27 was prepared in a 20% DMSO solution in 5 mM of Universal Buffer (CAPSO, TRIS, MES, sodium acetate, 5 mM each) to ensure the pH could be adjusted between 2 and 11 by addition of small quantities hydrochloric acid (2 M) or sodium hydroxide (2 M). Small quantities of hydrochloric acid or sodium hydroxide were added directly to the cuvette and the resulting pH was measured by a glass combination micro-electrode (MI-710, Microelectrodes Inc.) UV-Vis spectra were acquired using a PerkinElmer Lambda-35 instrument coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc.). The temperature was maintained at 20°C throughout the experiments. Spectra were baseline-corrected, assuming zero absorption at 850 nm. The resulting pH titration can be seen in Figure 29.

In comparison with 17, we immediately noticed a considerably higher pKₐ for 27. With a pKₐ of 8.2, 27 is almost 40 times more basic than 17 (pKₐ of 6.7). Furthermore, the absorption maximum for 27 (583 nm) is comparable to other TOM derivatives (545-600 nm, see Table 2).

While the absorption maximum for the azonium was 583 nm, the neutral compound has a wider absorption spectrum with a maximum at 378 nm, but a significant hump at 472 nm comparable to other TOM derivatives. Furthermore, 27 exhibited an isosbestic point between the neutral and azonium species at 489 nm.
Figure 29. pH titration of 27, note the formation of the red-shifted azonium at lower pH. 27 shows a pKₐ of 8.3 and an absorption maximum at 583 nm.

3.4 Photoisomerization of 27

After preparing 27, we set out to explore its photoisomerization, with the belief that a secondary aryl amine at the para position will affect the half-life of the cis isomer.

A solution of 27 was prepared in the Universal Buffer Mixture described in section 3.3. For each measurement, a quartz cuvette with a light path length of 10.00 mm was used. A deuterium lamp (Oriel, 200 A) was used as a source for the measuring beam while isomerization was achieved by irradiating with a red (Thor Labs, 633 nm, 24V, 0.477A, 55mW/in²) high power LED. Thermal relaxation rates were measured by monitoring absorbance after removal of the red light source. Relative absorbance values were recorded using a diode array (Ocean optics, 400-USB). The output of the detector was recorded immediately after triggering the red light off. Fitting the diode array signal vs. elapsed time to a monoexponential decay process gave the observed kinetic constants for thermal relaxation. The temperature of the sample was maintained at 20°C during all measurements using a temperature controlled cuvette holder (Quantum Northwest, Inc.).

Photoisomerization was accomplished in aqueous buffer solution containing 20% DMSO, pH 8.6 at 20°C. Through irradiation with red (633 nm) light, a change in absorbance intensity is seen, corresponding to a change in the concentration of trans 27 (see Figure 30). The half life of the
cis isomer was observed to be 8.2 seconds. At lower pH, the half life was observed to be shorter, corresponding to faster relaxation rates and a more transient cis isomer.

Figure 30. Photoswitching of 27 with 633 nm light in a 20% DMSO solution of 5 mM Universal Buffer at pH 8.6 at 20°C. Note the inhomogeneous irradiation resulting in a slower than expected formation of the cis isomer.

Figure 31. Relaxation of 27 after irradiation with 633 nm light in a 20% DMSO solution of 5 mM Universal Buffer at pH 8.6 at 20°C. A mono exponential fit was applied, showing a half-life for the cis isomer of approximately 7.9 seconds.
3.5 Discussion and Conclusions

While, we were successful in creating an azobenzene capable of photo switching with near-IR light under physiological conditions, we were aware of the lack of functional handles with which we could conjugate our azobenzene to a biomolecule (peptide, protein, bioactive small molecule, etc.). To address this concern, we successfully synthesized 27 a TOM azobenzene with two free amines on both ends of the azobenzene. Not only does 27 possess functional handles with which it may be conjugated to other molecules, but it also unique in that it is what we believe to be the first azobenzene containing both secondary aryl amines at the para positions and multiple electron donating groups at the ortho and meta position.

Previously, our lab and others have focused on azobenzenes containing amides or tertiary aryl amines at the para positions. The synthesis of 27 allowed us to study the spectral and physical properties of an azobenzene with a quite different para substituent.

We noticed the high pKₐ of 27 (8.2) and a maximum absorption at 583 nm, both of which are similar to other TOM azobenzenes (reported in Chapter 2.3). Thus, it would appear that our new conditions provide a direct route to coupling of bifunctional amines to azobenzene cores. This finding can be capitalized upon, by the direct synthesis of azobenzenes bearing both the DOM motif and functional handles in one step. Compounds such as 28, for example, could be created for ligation onto cysteines on peptide backbones (see Figure 32).

![Figure 32](image-url)  
Figure 32. Proposed synthesis of novel azobenzene crosslinker (28) for use in peptide control.
3.6 References


Appendices

Spectral data for synthesized compounds:

• $^1$H NMR
• $^{13}$C NMR
• Mass spectra
(E)-1,2-bis(6-methoxy-8-((S)-3-(methylsulfonyl)pyrrolidin-1-yl)-2,3
dihydrobenzo[b][1,4]dioxin-5-yl)diazene (17)

$^1$H NMR (500 MHz, Chloroform-$d$)

$^{13}$C NMR (125 MHz, Chloroform-$d$)
$^1\text{H} - ^{13}\text{C} \text{ HSQC (700 MHz, Chloroform-}d\text{)}$

$^1\text{H} - ^1\text{H \ COSY (700 MHz, Chloroform-}d\text{)}$
Mass Spectrum (ESI-Q-TOF)

H and C nuclei assignments

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 6.03 (s, 2H, H$_a$), 4.38-4.33 (m, 4H, H$_b$), 4.32-4.26 (m, 4H, H$_c$), 4.00-3.94 (m, 2H, H$_d$), 3.83 (s, 6H, H$_e$), 3.75-3.69 (m, 4H, H$_f$, H$_g$), 3.56-3.50 (m, 2H, H$_h$), 3.50-3.44 (m, 2H, H$_i$), 2.95 (s, 6H, H$_j$), 2.43 (m, 4H, H$_k$) ppm.

$^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 147.96 (C6), 138.93 (C4), 138.19 (C1 and C2), 129.24 (C3) 94.42 (C5), 64.89 (C7), 63.58 (C8), 61.74 (C13), 57.41 (C9), 50.28 (C11), 49.54 (C10), 38.79 (C14), 25.68 (C12) ppm.
(E)-1,2-bis(6-methoxy-8-(2-((dimethylamino)methyl)azetidin-1-yl)-2,3 dihydrobenzo\[b\][1,4]dioxin-5-yl)diazene (18)
\(^1\)H NMR (500 MHz, Chloroform-d)

\(^{13}\)C NMR (125 MHz, Chloroform-d)
$^1\text{H} - ^{13}\text{C}$ HSQC (700 MHz, Chloroform-$d$)

$^1\text{H} - ^1\text{H}$ COSY (700 MHz, Chloroform-$d$)
Mass Spectrum (ESI-Q-TOF)

H and C nuclei assignments

$^1$H NMR (500 MHz, Chloroform-d) $\delta$ 2.24 (s, 12H, Ha), 2.58 (d, 4H, He), 2.85 (septet, 2H, Hd), 3.69 (dd, 4H, Hb), 3.82 (s, 6H, Hf), 4.25-4.13 (m, 8H, Hg), 4.31-4.35 (m, 4H, Hc), 5.62 (s, 2H, Hh) ppm.

$^{13}$C NMR (125 MHz, Chloroform-d) $\delta$ 29.25 (C3), 45.66 (C1), 56.81 (C12), 58.16 (C4), 63.10 (C11), 63.63 (C2), 64.99 (C8), 89.88 (C6), 127.08 (C10), 138.69 (C9), 143.32 (C7), 149.12 (C5) ppm
1,2-bis(4-bromo-2,6-dimethoxyphenyl)diazene (26)

$^1$H NMR (500 MHz, Chloroform-$d$)

$^{13}$C NMR (125 MHz, Chloroform-$d$)
(E)- \text{N}^1,\text{N}^{11}\text{-(diazene-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis(hexane-1,6-diamine)} (27)

\text{\textsuperscript{1}H NMR} (500 MHz, Methanol-\textit{d}_4)

\text{\textsuperscript{13}C NMR} (125 MHz, Methanol-\textit{d}_4)
Mass Spectrum (ESI-Q-TOF)

[Diagram of a mass spectrum with peaks labeled and counts vs. mass-to-charge (m/z) axis]