Towards Red Light Control of Ion Channel Blockers

by

Catherine Victoria H. Collins

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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Abstract

Photoswitchable ion channel blockers reported previously have proven effective for modulating the activity of voltage-gated potassium (Kᵥ) channels. One limitation of previously reported azobenzene-based blockers was that the wavelength needed to promote trans-to-cis isomerization ranged from ultraviolet (UV) to blue. Light in this wavelength range poorly penetrates tissues and is cytotoxic, which limits the utility of these photoswitchable compounds for in vivo studies of Kᵥ channel activity. We designed, synthesized and ran preliminary patch-clamp experiments on analogous ion channel blockers bearing the tetra-ortho-methoxy (TOM) substitution pattern to bathochromically shift the absorption spectrum to permit long-wavelength photo-control. In combination with a bis-para-amino functionality, these blockers can form azonium ions under physiological conditions, which absorb red light strongly and undergo trans-to-cis isomerization. Red-light effectively penetrates tissues, allowing for in vivo studies of biological systems. We also report synthesis and initial characterization of a novel, further red-shifted dioxane ortho-methoxy (DOM) azobenzene substitution pattern.
Acknowledgments

First and foremost I would like to express my sincere gratitude to my advisor Prof. G. Andrew Woolley for his excellent support, guidance and inspiration throughout my Master’s degree. He is the epitome of a good mentor: patient, kind and motivating. His scientific curiosity is contagious. Thank you for fostering such a positive research environment to work in.

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The work reported in this dissertation includes contributions from several individuals. The patch clamp experiments were run by Dr. Michael Kienzler (University of Maine) in the Isacoff laboratory at the University of California Berkeley. The dibromo dioxane-ortho-methoxy (diBr-DOM, 15) starting material was synthesized by Mingxin Dong at the Beijing Institute of Biotechnology. Finally, the computational studies were run by Amir Babalhavaeji.
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<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Ångstrom, $10^{-10}$ m</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>acid association constant</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>$^{13}$C</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, $^2$H</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>DFT</td>
<td>density functional theory</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>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl, $\text{CH}_3\text{CH}_2$</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</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>
</tbody>
</table>
Hz  hertz, s⁻¹
IR  infrared
J  joule, kgm²s⁻²
J  coupling constant (in spectroscopy)
K  degree Kelvin
k  rate constant
Kₐ  acid dissociation constant
Kᵥ  voltage-gated potassium
L  litre, 10⁻³ m⁻³
λ  wavelength
LED  light emitting diode
LiHMDS  lithium hexamethyldisilazide
M  molar, molL⁻¹
m/z  mass-to-charge ratio (in mass spectrometry)
Me  methyl, CH₃-
MeOH  methanol
MES  2-(N-morpholino)ethanesulfonic acid
min  minutes
mol  mole, 6.022·10⁻²³ particles
MS  mass spectrum, mass spectrometry
NMR  nuclear magnetic resonance
Ph  phenyl, C₆H₅-
Q-TOF  quadrupole time-of-flight mass spectrometry
QA  quaternary ammonium
R  alkyl
Rf  retention factor
rt  room temperature
SPARK  synthetic photoisomerizable azobenzene-regulated K⁺ channels
τ₁/₂  half-life
TDDFT  time dependent density functional theory
TEA  tetraethylammonium
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran, $C_4H_4O$</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>TRPV1</td>
<td>transient receptor potential cation channel subfamily V member 1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
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Chapter 1
Introduction

1.1 Electrical Signaling and Voltage-Gated Potassium Channels

The movement of ions, mainly sodium, potassium, calcium and chloride, through ion channels results in electrical signalling in the nervous system. Ion channels have selective permeability, and therefore discriminate which ions can passively flow through the pore down the electrochemical gradient. The ability of a channel to open or close the pore in response to a change in voltage is called voltage-gating. Voltage-gated potassium (K\textsubscript{V}) channels play important roles in cell signalling through repolarizing, hyperpolarizing and helping to set the resting potential of the cell. They regulate the excitability of the cell by controlling the duration of action potentials and the ability of the cell to repetitively fire. Humans have 40 voltage-gated potassium channels which control diverse physiological processes in the body and are implicated in cancer, autoimmune diseases, as well as neurological and cardiovascular disorders.

Structurally, the pore of K\textsubscript{V} channels is formed by four membrane-spanning monomers, each with six α-helical transmembrane segments. These segments act as either voltage sensors (S1-S4) or selectivity filters (S5-S6) for K\textsuperscript{+} ions. K\textsubscript{V} channels are usually expressed with voltage-gated sodium and calcium channels and are closed at the resting potential of the cell of -40 mV to -100 mV. The intracellular concentration of potassium is 155 mM, 40-fold higher than the extracellular concentration. When the voltage of the cell gets above a certain threshold, the channels open allowing millions of K\textsuperscript{+} ions to efflux down the concentration gradient per second. Channel opening leads to repolarization, or even hyperpolarization, of the cell membrane and the subsequent closing of K\textsubscript{V} channels. Voltage-gated potassium channels have a remarkable ability to open, close and inactivate on millisecond time scales in response to changes in voltage. Some K\textsubscript{V} channels, such as Shaker B, undergo rapid inactivation since the N-terminal tail can block the pore. This inactivation is removed in mutated K\textsubscript{V} channels for a simplified model to study photo-control of channel currents. A second, slower, inactivation occurs when the C-terminus undergoes a conformational change. Mutations are often also induced to slow this type of inactivation. The voltage-gated potassium channel, K\textsubscript{V}2.1 was chosen for preliminary testing of our red-shifted ion channel blockers (detailed in Chapter 2). This channel is a delayed rectifier which is involved in neuronal repolarization of the action
potential. Brief depolarization activates the channel, whereas prolonged depolarization causes the channel to undergo slow inactivation. In pancreatic β-cells, Kv2.1 inhibition enhances insulin secretion and therefore could potentially have therapeutic benefits for type-2 diabetes mellitus.

1.2 Tetraethylammonium as a Channel Blocker

Voltage-gated potassium channels are targeted both in nature and by scientists to modulate channel activity. Natural toxins which act on ion channels are commonly short peptide sequences which are present in the venoms of a diverse set of animals such as scorpions, spiders, snakes, marine cone snails and sea anemones. While Kv channel blocking occurs in nature, there have also been small molecules designed by scientists to block channel activity. Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) are unselective Kv channel blockers (Figure 1). Under physiological conditions, they are cationic and block the inner pore of most open Kv channels.

**Figure 1.** Unselective Kv channel blockers TEA and 4-AP.

TEA and other tetraalkylammonium compounds block voltage-gated potassium channels from the intracellular side of the cell membrane. TEA bears a similar charge and radius to hydrated potassium ions and therefore is thought to mimic the ions. However, the inability of TEA to lose the ethyl groups, as potassium sheds water molecules, prevents it from crossing through the selectivity filter of the pore. Thus, the binding of TEA to the pore lining domain prevents potassium ions from crossing through the pore. The mechanism of TEA inactivation of the pore is called open-channel blocking since the block only functions when the channel is opened by a depolarizing pulse. The internal binding affinity of TEA for voltage-gated potassium channels was determined to be approximately 1 mM. While TEA blocks the inner pore of Kv channels, it has also been shown to be able to block externally in a subset of voltage-gated
potassium channels. Compounds which mimic the binding of TEA to block ion channels, such as lidocaine and its permanently charged derivative QX-314 (Figure 2), have been widely used as local anesthetics. These anesthetics prevent initiation of action potentials and silence neuronal activity by blocking voltage-gated sodium channels from the intracellular matrix.

![Lidocaine and QX-314](image)

**Figure 2.** Sodium channel blockers Lidocaine and QX-314.

### 1.3 Light Control of Ion Channel Blockers

Voltage-gated potassium channels are not natively light sensitive and therefore chemists and biologists can use optical tools in conjunction with the native channel to photo-regulate firing of the action potential. Azobenzenes are one of the most versatile and reliable photoswitches and are widely used to control biological systems. The ability of azobenzene to undergo reversible photoswitching was first discovered by Hartley in 1937 when he observed that irradiation with UV light produced the cis isomer and either irradiating with blue light or allowing thermal relaxation in the dark recovered the more stable trans isomer. Azobenzenes have several properties that make them well-suited for use *in vivo*. Firstly, azobenzenes have high molar extinction coefficients and high quantum yields which permits low intensity light to be used for photoisomerization. Secondly, the geometries of the trans and cis isomers are well-defined and the distance between bis-para-substituents changes substantially upon isomerization. Thirdly, azobenzenes are chemically inert under biological conditions. Lastly, substitutions to the azobenzene ring system can tune the absorption spectra and therefore the wavelengths of light necessary to photoisomerize the compounds. Kramer and colleagues took advantage of the extracellular quaternary ammonium (QA) binding site for photo-control of voltage-gated potassium channels *in vivo*. They used a maleimide-azobenzene-quaternary ammonium tether (MAQ, Figure 3), where the maleimide reacted with a cysteine residue engineered on the extracellular side of a Kv channel. The length of MAQ was guided by work by Blaustein *et al.*
which showed that the distance between the tether and the QA binding site at the pore opening was highly important. Photo-isomerization between the trans and cis isomers, which changes the length of the tethered QA, permitted control of neuronal activity in rat hippocampal neurons. These channels were named synthetic photoisomerizable azobenzene-regulated K⁺ (SPARK) channels. While this system permitted rapid, precise and reversible control of the firing of neurons, exogenous expression of the Cys-modified channels was necessary. To permit photo-control of the native Kᵥ channels, non-covalent azobenzene-based ion channel blockers were necessary. Kramer and Trauner developed blockers AAQ²⁹ and DENAQ³⁰ respectively (Figure 3), which blocked intracellular Kᵥ current in the trans conformation but not in the cis conformation. AAQ and DENAQ are membrane-permeable and therefore they diffused across the membrane to block Kᵥ channels intracellularly.

Figure 3. Structures of photochromic ion channel blockers MAQ, AAQ, DENAQ and QAQ.

Kramer and colleagues extended this work to develop quaternary ammonium-azobenzene-quaternary ammonium (QAQ, Figure 3) which contains two copies of the QX-314 anesthetic linked by a photoisomerizable azobenzene.³¹ Since QAQ is doubly charged, it cannot passively cross the cell membrane; thus Kramer and colleagues used a dilated pore protein to deliver QAQ to the cytoplasm where it functions as an ion channel blocker. QAQ functioning as an intracellular photochromic ion channel blocker is depicted in Figure 4. Quaternary ammoniums are not only able to block voltage-gated potassium channels, but also sodium and calcium channels; therefore the selectivity of these compounds in a given cell type or tissue where
multiple channel types are present must be tested on a case by case basis. QAQ was developed to target nociceptors, or pain sensing neurons: this application is detailed in Section 1.4.

Figure 4. (a) Photoisomerization of QAQ. (b) Internal trans-blocking and cis-unblocking of QAQ. Figure adapted from Fehrentz, T. et al. ChemBioChem 2012, 13, 1746-1749.

1.4 Photo-control of Nociception using QAQ

Nociceptors, or pain-sensing neurons, respond to noxious stimuli. There are three categories of noxious stimuli: thermal, mechanical and chemical. Nociceptors have a high density of ion channels which respond either directly or indirectly to noxious stimuli. Voltage-gated sodium and potassium channels conduct the nociceptor signal to downstream neurons which respond to the noxious stimuli. In chronic pain, the nociceptors are constantly relaying signals of pain in an unregulated manner. Thus, compounds that target nociceptors and reduce the excitability of these cells to prevent signal transmission could be of therapeutic benefit.

Local anesthetics work to suppress pain by blocking ion channels; however, they are limited in that they act non-selectively and therefore suppress the excitatory signals of all neurons. The
effects of anesthetics also wear off slowly since there is no mechanism to control their action and therefore the silencing persists until the compounds diffuse away or are metabolized by the cell.\textsuperscript{34} These limitations were both addressed by Kramer and colleagues in the design of QAQ.\textsuperscript{31} Since QAQ is a doubly charged compound, it cannot readily cross the cell membrane. They harnessed the capsaicin receptor (TRPV1) to transport QAQ selectively into nociceptive neurons using an approach previously described by Woolf and colleagues.\textsuperscript{23} TRPV1, which responds to noxious heat, is expressed almost exclusively in nociceptive neurons.\textsuperscript{35} When TRPV1 is activated for a prolonged period of time by the agonist capsaicin, it enters a pore-dilated state and relatively large cations, such as QAQ, are able to cross into the cell as a result. Once inside the neurons, irradiation with 380 nm UV light can isomerize QAQ from \textit{trans}-to-\textit{cis} and either 500 nm light or thermal relaxation permits isomerization back to the more stable \textit{trans} form (Figure 4a). \textit{Trans}-QAQ blocked voltage-gated sodium, calcium and potassium channels and resulted in a decrease in the electrical excitability of the neurons whereas the \textit{cis} isomer of QAQ unblocked the channels.\textsuperscript{31} Kramer and colleagues were able to use QAQ as a local anesthetic in the eyes of rats using capsaicin to dilate the TRPV1 receptor to deliver QAQ selectively into nociceptive neurons. Testing the blink sensitivity of the rats, it was seen that irradiation with 500 nm light decreased the blink response since the excitability of the neurons was blocked by \textit{trans}-QAQ.\textsuperscript{31} This observed decrease in the intrinsic excitability diminished when 380 nm light isomerized QAQ into the unblocking \textit{cis} isomer. Thus, the ability of the rats to sense pain in the nociceptors in their retinas was modulated by photo-control of QAQ.

1.5 Red-Shifted Azobenzene Photoswitches

While QAQ provides an example of a photochromic ligand that can be used to modulate the electrical excitability in nociceptors, the \textit{in vivo} utility of QAQ as a local anesthetic is limited by the wavelengths of light necessary to isomerize the azobenzene. UV light, which permits \textit{trans}-to-\textit{cis} isomerization of QAQ, is phototoxic and damaging to cells.\textsuperscript{36-37} Longer wavelengths of light are less phototoxic and allow for much greater penetration into tissue samples. The optical absorption in biological tissue samples comes mainly from the presence of water, hemoglobin and melanin.\textsuperscript{38} The absorption of these compounds creates an optical window in the red, far red
or optimally near-infrared (IR) region where light most effectively penetrates into tissues.\textsuperscript{38-39} Unmodified azobenzene undergoes \textit{trans}-to-\textit{cis} isomerization under UV light where the penetration of tissue is low. Thus, QAQ was limited to photo-control the excitability of nociceptors in the retina. To increase the utility of azobenzene photoswitches for use \textit{in vivo}, much work has been done to red-shift the absorption spectrum so azobenzenes absorb light in a wavelength range that is optimal for tissue penetration. The absorption spectrum of azobenzene is highly dependent on both the type of aromatic ring and the nature and position of the substituents.\textsuperscript{40} Amino substituents at either the \textit{ortho} or \textit{para} positions were found to red-shift the absorption spectrum, especially when incorporated into ‘push-pull’ systems where one ring has an electron donating group and the other has an electron withdrawing group.\textsuperscript{41} A drawback is that red-shifted absorbances of azobenzene derivatives often lead to increased thermal relaxation rates.\textsuperscript{42} Aside from changing the substituents on the azobenzene ring system to increase the wavelength of absorption, there have been bridged azobenzenes, heterocycles, BF\textsubscript{2} adducts and two-photon chromophore systems, which also promote long wavelength absorption.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures.png}
\caption{Examples of a C2 bridged azobenzene (a), an azoheterocyclic switch (b), a BF\textsubscript{2} adduct (c) and a two-photon absorbing photoswitch (d).}
\end{figure}

The addition of a bridge between the C2 positions gave a large gap between the \(n-\pi^*\) transitions of the \textit{trans} and \textit{cis} forms that promoted high conversion between the two isomers since the spectral overlap was minimal.\textsuperscript{43} The \textit{cis} isomer was the more stable isomer compared to most azobenzenes where the \textit{trans} isomer is thermodynamically preferred. Woolley and colleagues
added bis-para-acetamido substituents to the bridged azobenzene core for conjugation and photo-control of peptide conformation (Figure 5a).\textsuperscript{44} Adding bis-para-amino substituents to the C2 bridged system, as shown by Herges and colleagues, red-shifted the absorption spectrum to allow 600 nm light to promote photoisomerization.\textsuperscript{45} While heterocyclic azo functionalities are common in dyes, their use as photoswitches has only been more recently explored.\textsuperscript{46-48} Heterocyclic azobenzenes demonstrate separated absorbance maxima of the two isomers and long cis thermal half-lives and therefore they may be useful scaffolds for robust photoswitching. Recently Fuchter and colleagues showed that the basic imidazole nitrogens in azoheteroarenes could better stabilize protonation in the cis versus the trans isomer which increased the thermodynamic stability and pK\textsubscript{a} of the cis form (Figure 5b).\textsuperscript{48} It was recently discovered that BF\textsubscript{2}-adducts of azobenzenes can have transitions in the far-red and near-IR region of the spectrum (Figure 5c); however, these compounds are quite susceptible to hydrolysis.\textsuperscript{49-50} Lastly, two photon absorption cross sections can also be used to promote near-IR photoswitching of bioactive azobenzene compounds (Figure 5d).\textsuperscript{51-52} Design of these compounds can enhance the ability to absorb two photons, such as incorporating naphthalene,\textsuperscript{51} pyrene\textsuperscript{53} or triarylamino\textsuperscript{54} as the antenna to sensitize the two photon absorption cross section. These antennas increase the structural complexity and size of the photoswitches which may limit their in vivo utility.

\textbf{Figure 6.} Woolley and coworkers’ various azobenzenes bearing different \textit{para}-substituents and \textit{tetra-ortho} substituents to red-shift the absorbance wavelengths.

To avoid these limitations, Woolley and coworkers have worked to optimize small and symmetric azo compounds which can be tuned to undergo long wavelength photoswitching by modifying the substitution pattern on the azobenzene rings. Initially, bis-para-amido substituents
were added to the ring system (Figure 6a).\textsuperscript{55} Various ortho-amino substituents were also tested and an example is depicted in Figure 6b; however, increased photobleaching was observed with the more electron rich azo systems. Changing the ortho-amino groups to tetra-ortho-methoxy (TOM) substituents permitted the use of green light for trans-to-cis isomerization due to a red-shifted n-π\textsuperscript{*} transition (Figure 6c).\textsuperscript{56} Tetra-ortho-chloro\textsuperscript{57} and tetra-ortho-thioether\textsuperscript{58} substituted azobenzene rings also displayed promising red-shifted absorption properties. Hecht and coworkers observed that the tetra-ortho-fluoro substitution, while not as red-shifted as the tetra-ortho-methoxy substituted azobenzene, had a very slow thermal relaxation rate and therefore was bistable on biological time scales.\textsuperscript{59} The tetra-ortho-chloro substituted azobenzene was incorporated into a long wavelength absorbing photoswitchable tethered ligand for the ionotropic glutamate channel in collaboration with the Isacoff group.\textsuperscript{60} The TOM substitution pattern was utilized as a photoswitch to control the helicity of a peptide in zebrafish embryos.\textsuperscript{57} A similar azobenzene bearing the TOM substitution pattern with thiol-reactive bis-para-maleimides was crosslinked to a peptide and permitted photo-control of the peptide helical conformation with 617 nm light.\textsuperscript{61} Changing the bis-para-amido to bis-para-amino substituents (Figure 6d) was found to further red-shift the absorbance band since the azo bond was able to be protonated at neutral pH.\textsuperscript{62} Usually azonium formation occurs at a pH of less than 3.5 and therefore azobenzenes are not protonated under physiological pH.\textsuperscript{63} Azonium ions undergo rapid cis-to-trans thermal relaxation (2-3 μs), and therefore bright light sources are necessary to push the equilibrium towards the cis form.\textsuperscript{64} The TOM-substituted azonium, however, underwent thermal relaxation approximately a million times slower.\textsuperscript{62} It was hypothesized that this slower rate of thermal relaxation was due to a difference in pK\textsubscript{a} of the trans and cis isomers since stabilization of the azonium through H-bonding interactions with an ortho-methoxy substituent occurred only in the trans isomer. Thus, photoisomerization to cis was coupled to loss of the proton and the increase in double bond character of the azo bond slowed the rate of thermal relaxation. Fast-relaxing azobenzenes have previously been used to photo-control the ionotropic glutamate receptor.\textsuperscript{65} Red-light control of a TOM-substituted azobenzene with bis-para-piperazines linked to a peptide underwent isomerization in whole blood using 635 nm light.\textsuperscript{62} Modifying the nature of the substitutions on the aromatic rings of the azobenzene therefore allows substantial control over the absorption spectra and the wavelengths necessary to promote trans-to-cis isomerization.
The red-shifted azobenzenes have also shown promise for their utility in \textit{in vivo} systems, especially due to the increased ability of long wavelength light to penetrate into tissues.

1.6 Red-Shifted Ion Channel Blockers

To red-shift the wavelength necessary to control voltage-gated ion channels, Trauner and colleagues made red-shifted analogues of the photochromic blocker QAQ by adding \textit{ortho}-substitutions on the azo core.\textsuperscript{32} The furthest red-shifted compound identified that was able to modulate voltage-gated ion channel activity bore an \textit{ortho}-methoxy substituent on either side of the azo functionality (2,2'-dimethoxy-QAQ, Figure 7). Photochromic ligands bearing larger \textit{ortho} substituents, such as 2,2'-N-dimethylpiperazino and 2,2'-dimethylamino (Figure 7), were not able to modulate the activity of the voltage-gated K\textsuperscript{+} channels. It was hypothesized that this was potentially due to steric hindrance within the confined inner cavity of the ion channel. The 2,2'-dimethoxy-QAQ compound had a red-shifted absorption spectrum compared to QAQ and \textit{trans}-to-\textit{cis} isomerization occurred under 420 nm light instead of 380 nm light.\textsuperscript{32} While this was an improvement to the original photochromic ligand QAQ, 420 nm light still is far below the wavelengths which are necessary for adequate tissue penetration for \textit{in vivo} studies.

Using the approaches for further red-shifting of azobenzenes designed by Woolley and coworkers, we proposed to synthesize two substituted analogues of QAQ that would have further red-shifted absorbances than previously studied derivatives. Figure 7 depicts the open-channel blockers QAQ and 2,2'-dimethoxy-QAQ as well as the proposed structures of red-shifted analogues TOM-QAQ and TMP-QAQ. TOM-QAQ bears the red-shifted TOM substitution pattern in combination with the bis-\textit{para}-amides to be analogous to the structure of QAQ aside from the added electron-donating ring substituents. Since Woolley and colleagues also reported previously that a bis-\textit{para}-amino in conjunction with the TOM substitution pattern greatly red-shifts the absorption spectrum due to the ability to form the protonated azonium ion under physiological conditions.\textsuperscript{62} Therefore, we proposed to make a TMP-QAQ derivative with the bis-\textit{para}-piperazine linker between the azobenzene and the quaternary ammonium, as depicted in Figure 7. This derivative would have the furthest red-shifted absorption spectrum of an open-
channel blocker for the voltage-gated potassium channel to date, should it be able to modulate the channel activity. The tolerance of the Kv channel for the red-shifted channel blockers bearing the TOM substitution pattern, as well as the additional bis-para-piperazine linkers, is of interest. Synthesis, photochemical characterization and preliminary in vivo studies of TOM-QAQ and TMP-QAQ as open-channel blockers are reported in Chapter 2.

Figure 7. Photochromic open-channel blockers of Kv channels QAQ and 2,2′-dimethoxy-QAQ (top). Sterically hindered derivatives 2,2′-N-dimethylpiperazine-QAQ and 2,2′-dimethylamino-QAQ which did not modulate Kv channel current (middle). Proposed red-shifted analogues TOM-QAQ and TMP-QAQ (bottom).

1.7 A Novel Red-Shifted Azobenzene Scaffold

Azobenzenes bearing the tetra-ortho-methoxy (TOM) substitution pattern in conjunction with a bis-para-amino group have desirable red-shifted absorption bands, especially when in the protonated azonium form. The best optical window for photoswitching in biological systems,
however, occurs in the near-IR so there is continued interest in further red-shifting the azobenzene core. This would maximize the optical control of azobenzene photoswitches in biological systems where the ability of light to penetrate into tissue is the limiting factor. The ability of the TOM-substituted azobenzenes with para-amino groups to be protonated to form the azonium species greatly red-shifts the $\lambda_{\text{max}}$ of the trans isomer. The azonium is stabilized by both the increased electron density of the ring and the proximity to the ortho-methoxy substituents for H-bonding. Wizinger and colleagues found that positioning a weak auxochrome, such as a methoxy substituent, para to the ortho-methoxy substituent on azo compounds caused a bathochromic shift. This was consistent with the law of distribution of auxochromes which was first recognized by Kugel and coworkers. Woolley and colleagues used this rule to design further red-shifted azobenzene photoswitches. By retaining the ortho-methoxy substitution for intramolecular H-bonding and adding a meta-methoxy substituent, significant absorbance in the far-red and near-IR was observed, further shifted than that observed with the TOM substitution pattern. One limitation to the ortho-meta substitution pattern was the rapid thermal relaxation observed. Compared to the tetra-ortho-methoxy substituted azobenzenes, this thermal relaxation was 1000 times faster. A slower thermal relaxation is ideal to allow a greater proportion of the cis isomer to form under irradiation with dim light sources for a larger amount of photo-control. Therefore, to retain both the bathochromic shift and the slower thermal relaxation time, both tetra-ortho-methoxy and meta-methoxy substitutions need to be retained. Thus, we designed the dioxane ortho-methoxy (DOM) substitution pattern (Figure 8). We hypothesized that restricting the ortho and meta substituents into a dioxane ring would minimize steric hindrance with the para-amino group for optimal electron donation into the ring system and minimize steric hindrance to promote intramolecular H-bond formation with the azonium ion. TDDFT calculations (B3LYP (6-31++G**)) suggested that the $\lambda_{\text{max}}$ of the trans isomer bearing the DOM substitution pattern would be further red-shifted than the previously studied TOM substitution pattern. To study the red-shifting of the DOM-substituted azobenzenes compared to TOM analogues, we varied the nature of the bis-para-amino substituents to see the effects on the photochemical properties. The behaviour of the novel DOM scaffold, in comparison to the TOM scaffold, is described in Chapter 3.
Figure 8. Tetra-ortho-methoxy (TOM), ortho-meta-methoxy and dioxane-ortho-methoxy (DOM) substitution patterns.

1.8 References


Chapter 2
Towards Red-Light Control of Ion Channel Blockers

2.1 Methods

General

All commercial materials (solvents, reagents and substrates) were used as received. SiliaFlash P60 silica gel of particle size 40-63 μm was used for column chromatography (Silicycle Inc.) 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 spectra were recorded on the Varian UnityPlus 500 MHz or Varian Mercury 400 MHz spectrometer. 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-$d_4$ (3.31 ppm), DMSO-$d_6$ (2.50 ppm), deuterium oxide (4.79 ppm) and acetone-$d_6$ (2.05 ppm). For $^{13}$C NMR: chloroform-$d$ (77.16 ppm), methanol-$d_4$ (49.00 ppm), DMSO-$d_6$ (39.52 ppm) and acetone-$d_6$ (29.84, 206.26 ppm). $^1$H NMR spectral characterization is reported as follows: chemical shift (multiplicity, coupling constant, integration, assignment, isomer (if necessary)). The adjacent quadrupolar $^{14}$N nuclei may be contributing to missing sp$^2$ hybridized $^{13}$C resonances. 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.
Scheme 1. Synthesis of 4\(^a\).

\[ \text{HO} \text{NO}_2 \text{OH} \xrightarrow{\text{a}} \text{O} \text{NO}_2 \text{O} \xrightarrow{\text{b}} \text{O} \text{NH}_2 \text{O} \]

\[ \text{O} \text{O} \text{Br} \text{Br} \text{N} \xrightarrow{\text{d}} \text{O} \text{NH}_2 \text{O} \]

\(^a\)Conditions and reagents: (a) Methyl iodide, K\(_2\)CO\(_3\), DMF, rt, 5 h, yield quantitative; (b) Iron filings, NH\(_4\)Cl, H\(_2\)O/MeOH, 80 °C, 12 h, yield 74%; (c) Bromine, DCM, 0 °C, 40 min, yield 65%; (d) KMnO\(_4\), CuSO\(_4\)·5H\(_2\)O, DCM, reflux, 40 h, yield 40%.

1,3-dimethoxy-2-nitro-benzene (1)

To a solution of 2-nitroresorcinol (1.55 g, 10 mmol) and anhydrous potassium carbonate (4.1 g, 30 mmol) in anhydrous DMF (25 mL) was added CH\(_3\)I (1.9 mL, 30 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 solids, 1.87 g, yield quantitative). \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 3.86 (s, 6H, OCH\(_3\)), 6.62 (d, \(J = 8.5\) Hz, 2H, Ar), 7.32 (t, \(J = 8.5\) Hz, 1H, Ar). \(^{13}\)C NMR (126 MHz, Chloroform-\(d\)) \(\delta\) 56.43, 104.49, 131.10, 151.82. ESI-DART: \(m/z\) calc. for C\(_8\)H\(_9\)NO\(_4\): 184.05 [M+H]\(^+\); found 184.06.

2,6-dimethoxyaniline (2)

To a vigorously stirring solution of 1 (1.7 g, 9 mmol) and ammonium chloride (3.4 g, 56 mmol) in water/methanol (1:5), iron filings (1.7 g, 30 mmol) were added and the solution was heated to a reflux for 12 h. The reaction was cooled to room temperature, diluted with water then the methanol was removed \textit{in vacuo}. The aqueous solution was extracted with DCM and 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 white crystals (1.1 g, yield 74%). $^1$H NMR (500 MHz, Chloroform-$d$) δ 3.86 (s, 6H, OCH$_3$), 6.52 (m, 2H, Ar), 6.70 (m, 1H, Ar). $^{13}$C NMR (126 MHz, Chloroform-$d$) δ 55.82, 104.07, 117.07, 125.16, 147.61. ESI-DART: m/z calc. for C$_8$H$_{11}$NO$_2$: 154.1 [M+H]$^+$; found 154.1.

2,6-dimethoxy-4-bromo-aniline (3)

A solution of bromine (0.15 mL, 5.9 mmol) in DCM (5 mL) was slowly added to a solution of 2 (900 mg, 5.9 mmol) in DCM (50 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 (887 mg, yield 65%). $^1$H NMR (500 MHz, Chloroform-$d$) δ 3.75 (s, 6H, OCH$_3$), 6.63 (s, 2H, Ar). $^{13}$C NMR (126 MHz, Chloroform-$d$) δ 55.95, 107.60, 108.48, 124.71, 147.60. ESI-DART: m/z calc. for C$_8$H$_{10}$BrNO$_2$: 231.99 [M+H]$^+$; found 232.00.

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

To a solution of 3 (0.5 g, 2 mmol) in DCM (40 mL) was added KMnO$_4$ (1.2 g) and CuSO$_4$·5H$_2$O 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 (200 mg, yield 40%). $^1$H NMR (399 MHz, DMSO-$d_6$) δ 3.58 (s, 5.5H, OCH$_3$, cis), 3.75 (s, 12H, OCH$_3$, trans), 6.79 (s, 1.9H, Ar, cis), 7.01 (s, 4H, Ar, trans). Ratio *trans:*cis (68:32). ESI-Q-TOF: m/z calc. for C$_{16}$H$_{16}$Br$_2$N$_2$O$_4$: 458.95 [M+H]$^+$; found 458.95.
Scheme 2. Synthesis of 6².

*²Conditions and reagents: (a) i. Et₃N, EtOH, 100 °C, 5 h, ii. Et₃N, H₂O, 100 °C, 4 h, yield 75%; (b) Oxalyl chloride, ACN/DCM, DMF (cat.), rt, 1 h, not isolated.

N-(carboxymethyl)-N,N-diethylethanaminium bromide (5)

A mixture of methyl bromoacetate (310 μL, 3.3 mmol) and triethylamine (560 μL, 4 mmol) in ethanol was heated to a reflux for 5 h. The solvent was removed *in vacuo* and then it was taken up in water and triethylamine (2 mL, 16 mmol). The solution was heated to a reflux for 4 h, cooled, and then the solvent was removed *in vacuo*. After recrystallization in ethanol, the pure product was obtained as white crystals (1.5 g, 75%). ¹H NMR (500 MHz, Methanol-d₄) δ 1.31 (t, J = 7.3 Hz, 9H, CH₃), 3.60 (q, J = 7.3 Hz, 6H, CH₂), 3.95 (s, 2H, CH₂). ¹³C NMR (126 MHz, Methanol-d₄) δ 6.41, 53.40, 56.01, 166.63. ESI-Q-TOF: *m/z* calc. for C₈H₁₈NO₂⁺: 160.1 [M⁺]; found 160.1.

2-chloro-N,N,N-triethyl-2-oxoethanaminium chloride (6)

To a solution of 5 (70 mg, 0.45 mmol) suspended in anhydrous ACN under nitrogen gas, oxalyl chloride (46 μL, 0.54 mmol) in anhydrous DCM was added dropwise. A drop of DMF was added and the reaction was stirred at room temperature for 1 h. The solvent was removed *in vacuo* and then the product was dried under vacuum for 1 h to yield a yellow oil which was used directly in subsequent coupling reactions.

\[ \text{Conditions and reagents: (a) i. LiHMDS, Pd\(_2\)(dba)\(_3\), P(t-bu)\(_3\), toluene, rt, 16 h, ii. HCl, yield 90%; (b) 6, DIPEA, DMF, 0 °C to rt, 18 h, yield 50%.} \]

4,4’-(diazene-1,2-diyl)bis(3,5-dimethoxyaniline) (7)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 4 (25 mg, 0.05 mmol), Pd\(_2\)(dba)\(_3\) (9 mg, 0.01 mmol), and LiHMDS (33.5 mg, 0.2 mmol), followed by toluene (3 mL) and P(tBu)\(_3\) (20 μL (1 M in toluene), 0.02 mmol). The tube was capped with a Teflon stopper and stirred at room temperature for 16 h. The crude reaction mixture was diluted with Et\(_2\)O (20 mL), and the silylamide was deprotected with one drop of aqueous 1 M HCl. In a separatory funnel, the organics was washed with aqueous 1 M NaOH and brine. The organic layer was dried over Na\(_2\)SO\(_4\), filtered, and the solvent was removed in vacuo. Column chromatography (methanol:DCM (1:6)) yielded the semi-pure product as a red solid which was used directly in the following step (16 mg, yield 90%). \(^{1}\)H NMR (500 MHz, Methanol-\(d_4\)) δ 3.34 (s, 12H), 6.11 (s, 4H). ESI-Q-TOF: \(m/z\) calc. for C\(_{16}\)H\(_{21}\)N\(_4\)O\(_4\): 333.15 [M+H]\(^+\); found 333.15.

2,2’-((diazene-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis(azanediyl))bis(N,N,N-triethyl-2-oxoethanaminium) (8)

In a flask, flushed with nitrogen gas, 7 (18 mg, 0.05 mmol) was dissolved in anhydrous DMF and basified with DIPEA (139 μL, 0.8 mmol). The solution was stirred at room temperature for 10 min and then cooled to 0 °C. A solution of 6 (39 mg, 0.2 mmol) in DMF was added dropwise and
then the reaction was warmed to room temperature and stirred for 18 h. The solvent was removed \textit{in vacuo} and then the product was purified by reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5\% to 70\% ACN/water containing 0.1\% trifluoroacetic acid over 25 min) yielding a red solid (yield 50\% by HPLC). \(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 1.37 (t, 6.1H, CH, \textit{cis}), 1.40 (t, 18H, CH, \textit{trans}), 3.64 (q, 4.2H, CH, \textit{cis}), 3.66 (s, 4.2H, OCH, \textit{cis}), 3.70 (q, 12H, CH, \textit{trans}), 4.01 (s, 12H, OCH, \textit{trans}), 4.17 (s, 1.4H, CH, \textit{cis}), 4.28 (s, 4H, CH, \textit{trans}), 6.96 (s, 1.4H, Ar, \textit{cis}), 7.25 (s, 4H, Ar, \textit{trans}). Ratio \textit{trans}:\textit{cis} (74:26). \(^{13}\)C NMR (126 MHz, Methanol-\(d_4\)) \(\delta\) 6.54, 54.43, 55.61, 66.71, 96.47, 153.90. ESI-Q-TOF: \textit{m}/\textit{z} calc. for C\(_{32}\)H\(_{52}\)N\(_6\)O\(_6\)^{2+}: 308.1969 [M^{2+}/2]; found 308.1973.

\textbf{Scheme 4.} Synthesis of 11\(^\alpha\).

\(^\alpha\)Conditions and reagents. (a) 1-Boc-piperazine, Cs\(_2\)CO\(_3\), Pd\(_2\)(dba)\(_3\), RuPhos, toluene, 100 \textdegree C, 17 h, yield 72\%; (b) TFA, DCM, 0 \textdegree C to rt, 1.5 h; (c) 6, K\(_2\)CO\(_3\), DMF, 0 \textdegree C to rt, 14 h, yield 20\%.
Di-tert-butyl 4,4’-(diazen-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis(piperazine-1-carboxylate) (9)

In an oven-dried pressure tube, cooled under nitrogen gas, 4 (50 mg, 0.11 mmol), 1-Boc-piperazine (61 mg, 0.33 mmol), Cs₂CO₃ (100 mg, 0.33 mmol), Pd₂(dba)₃ (18 mg, 0.02 mmol), and RuPhos (18 mg, 0.04 mmol) were combined. Toluene (3 mL) was added, the vessel was capped with a Teflon stopper and then heated to 100 °C to stir for 17 h. The reaction mixture was cooled to room temperature, diluted with water and extracted with ethyl acetate. The combined organics were washed with brine, dried with Na₂SO₄ and the solvent was removed in vacuo. Column chromatography (methanol:DCM (1:6)) yielded the pure product as a blue solid (53 mg, yield 72%). ¹H NMR (500 MHz, Acetone-d₆) δ 1.47 (s, 18H, CH₃), 3.45 (s, 8H, CH₂), 3.56 (s, 8H, CH₂), 3.91 (s, 12H, OCH₃), 6.38 (s, 4H, Ar). ¹³C NMR (126 MHz, Acetone-d₆) δ 27.64, 48.06, 56.30, 79.03, 93.11, 154.08. Note: The adjacent quadrupolar ¹⁴N nuclei may be contributing to missing sp² hybridized ¹³C resonances and observed resonances are consistent with published spectra.¹ ESI-DART: m/z calc. for C₃₄H₅₀N₆O₈: 671.4 [M+H]⁺; found 671.3.

1,2-bis(2,6-dimethoxy-4-(piperazin-1-yl)phenyl)diazene (10)

In a flask, 9 (20 mg, 0.03 mmol) was dissolved in DCM (3 mL) and cooled to 0 °C. TFA (0.4 mL) was added dropwise to make a 10% solution then the reaction was warmed to room temperature and set to stir for 1.5 h. The solvent was removed in vacuo and the product was dried under high vacuum. The crude product (14 mg) was used directly in the next reaction. ESI-Q-TOF: m/z calc. for C₂₄H₃₄N₆O₄: 471.3 [M+H]⁺; found 471.3.
2,2'-(4,4'-(diazene-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis(piperazine-4,1-diyl))bis(N,N,N-triethyl-2-oxoethanaminium) (11)

In a flask, 10 (14 mg, 0.03 mmol) was suspended in anhydrous DMF (1.5 mL) and treated with potassium carbonate (55 mg, 0.4 mmol). The solution was stirred for 15 min and then cooled to 0 °C. To the reaction, 6 (16 mg, 0.09 mmol) in anhydrous DMF (0.5 mL) was added dropwise and then the reaction was warmed to room temperature and stirred for 14 h. The solvent was removed in vacuo and reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid (yield 20% by HPLC).

$^1$H NMR (500 MHz, Deuterium Oxide) δ 1.18 (t, $J$ = 7.3 Hz, 18H, CH$_3$), 3.34 (m, 8H, CH$_2$), 3.52 (q, $J$ = 7.2 Hz, 12H, CH$_2$), 3.61 (m, 8H, CH$_2$), 3.91 (s, 12H, OCH$_3$), 4.19 (s, 4H, CH$_2$), 6.02 (s, 4H, Ar). $^{13}$C NMR (126 MHz, Deuterium Oxide) δ 6.95, 53.89, 54.47, 55.43, 57.43, 89.75. Note: TFA resonances observed at δ 116.26 (q), 162.91 (q). ESI-DART: $m/z$ calc. for C$_{40}$H$_{66}$N$_8$O$_6$:$^{2+}$: 377.2547 [M$^{2+}$/2]; found 377.2550.

**Sample preparation for UV-Visible spectroscopy**

Solutions were prepared in universal buffer with a mixture of four components (CAPSO, TRIS, MES, sodium acetate, 5 mM each) to ensure the pH could be easily adjusted between 2 and 11 by addition of small quantities of concentrated hydrochloric acid or sodium hydroxide. The pH of each solution was measured by a glass combination micro-electrode (MI-710, Microelectrodes Inc.). The compounds were dissolved in DMSO and then diluted with buffer with the DMSO percentage in the final solutions being 5%.

**Photoswitching of TOM-QAQ (8)**

A solution of 8 was prepared as described above and the pH was adjusted to 7.4. For each measurement, a quartz cuvette with a light path length of 10 mm was used. 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
 experiment. All spectra were baseline-corrected, assuming zero absorption at 800 nm. Various LEDs were used to drive photoisomerization of 8: blue LED (Thor Labs, 450 nm), green LED (LedEngin LZ4-41G100, 700 mA, 537 nm, 64 mW/cm²), amber LED (LedEngin LZ4-40A100-0000, 700 mA, 591 nm, 30mW/cm²), red LED (Thor Labs, 617 nm) and a UV LED (LedEngin, LZ4-40U600, 700 mA, 365 nm). The UV-Vis spectral data obtained for 8 after irradiation at various wavelengths of light are presented in Figure 10.

**pH-dependence of absorption spectra of TMP-QAQ (11)**

The solution of 11 was prepared as previously described. Small quantities of concentrated hydrochloric acid or sodium hydroxide were added directly to the 1 cm quartz cuvette and the pH was measured by a glass combination micro-electrode (MI-710, Microelectrodes Inc.) UV-Visible 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 experiment. All spectra were baseline-corrected, assuming zero absorption at 800 nm. HypSpec2014 (version 1.2.31)²⁻³ was used to calculate the apparent pKₐ for the trans form of 11 using the UV-visible spectral data and the corresponding pH. These data are presented in Figure 12.

Constants are reported as log(β) by HypSpec. To determine the pKₐ, the following equations were used, where A represents the parent neutral azobenzene, β is the acid association constant and Kₐ is the acid dissociation constant.

For a single protonation event (azonium formation):

\[ A + H^+ \rightleftharpoons AH^+ \]

\[ K_{a1} = \frac{[AH^+]}{[A][H^+]} \]

\[ \beta_1 = \frac{1}{K_{a1}} \]

\[ \log(K_{a1}) = -\log(\beta_1) \implies pK_{a1} = \log(\beta_1) \]

**Thermal relaxation kinetics for TMP-QAQ**

A solution of TMP-QAQ (11) was prepared in the buffer mixture described above. 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, 64 mW/cm²) or a blue LED (Thor Labs, 420 nm) was used as a source for the measuring beam while isomerization was achieved by irradiating with a red (Thor Labs, 617 nm) 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 photomultiplier tube (Oriel, Newport Corporation) 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. These data are shown in Figure 13 and Figure 14. Kinetic constants were the average of three decay curves. The temperature of the sample was maintained at 20 °C during all measurements using a temperature controlled cuvette holder (Quantum Northwest, Inc.). To confirm that the rate of cis-to-trans conversion was not affected by the wavelength of the measuring beam, relaxation rates were measured at two different wavelengths of the incident beam.

**Electrophysiological characterization of Kv2.1 for QAQ, TOM-QAQ and TMP-QAQ**

HEK-293 cells were cultured under standard conditions (DMEM containing 10% FBS). Cells were plated on poly-L-lysine (0.1 mg/mL) treated coverslips for electrophysiological measurements. HEK-293 cells were transfected with a Kv2.1 channel (pRBG4 DNA; CMV promoter) and Lipofectamine 2000 (Invitrogen) as the transfection agent. GFP was co-transfected as a fluorescent marker protein to identify transfected cells. Cells were measured after 24-48 h. Stock solutions of QAQ (1), TOM-QAQ (2) and TMP-QAQ (3) were made in anhydrous DMSO (~100 mM) and stored at -20 °C. Briefly, whole-cell recordings with HEK cells were performed on inverted microscopes (Olympus IX) using Axopatch 200B headstage/amplifiers (Molecular Devices). Experiments were performed at room temperature in voltage-clamp mode, typically at -80 mV. External bath solution contained: 138 mM NaCl, 1.5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 5 mM HEPES and 10 mM glucose at pH=7.4.
Patch pipettes were pulled from borosilicate glass to give 4-12 MΩ resistance when filled with internal solution pipette solution which contained: 10 mM NaCl, 135 mM K\(^+\) gluconate, 10 mM HEPES, 2 mM MgCl\(_2\), 2 mM MgATP, and 1 mM EGTA at pH=7.4 or 5.9. Photo-switching was performed using a Polychrome V Xe-lamp light source (15 nm FWHM bandwidth, Till Photonics), controlled through the Clampex software (Molecular Devices). This lamp allowed irradiation of the cells with wavelengths between 340 nm and 680 nm. For direct internal application through the patch pipette, the following concentrations were used: 100 μM QAQ, 400 μM TOM-QAQ and 100 μM TMP-QAQ. Measurements were started after 5 min of equilibration time. Data were analyzed using Clampfit 10.4 (Molecular Devices). These data are presented in Figure 15, Figure 16 and Figure 17.

2.2 Results and Discussion

Two analogues of the open-channel blocker QAQ, TOM-QAQ (8) and TMP-QAQ (11), were synthesized, the spectral properties were analyzed and preliminary testing in vivo against the voltage-gated potassium channel K\(_V\)2.1 were conducted.

To access TOM-QAQ and TMP-QAQ, both analogues were synthesized from the tetra-ortho-methoxy substituted dibromodiazene intermediate (4). The precursor 4 was made via the synthetic route described in Scheme 1. Starting from 2-nitroresorcinol, dimethylation with methyl iodide under basic conditions yielded 1. The nitro group was then reduced to the amine over iron filings in a solution of aqueous ammonium chloride and methanol to yield the dimethoxyaniline (2). Bromination of 2 was achieved under standard conditions with Br\(_2\) to afford the para-bromoaniline (3). To yield the desired diazene (4), potassium permanganate and copper sulfate were utilized to avoid a reductive step.\(^4\)

To produce the triethylammoniumacetyl chloride (6), as shown in Scheme 2, synthetic procedures previously described were harnessed. Starting from methyl bromoacetate, triethylamine was used as the nucleophile to replace the α-bromo substituent.\(^5\) Initially, acidic conditions were used to deprotect the methoxy ester; however, the deprotection was not complete. During reflux in triethylamine and ethanol, we found the methoxy ester was replaced with an ethoxy ester since ethanol was used as the solvent. Based on the ease of this exchange,
the ethoxy ester was deprotected by refluxing the product in triethylamine and water to yield the desired triethylammoniumacetic acid (5). The acid chloride was subsequently generated as previously described using oxalyl chloride to generate triethylammoniumacetyl chloride (6) which was used directly in subsequent coupling reactions without further purification. 

TOM-QAQ (8) was synthesized from the tetra-ortho-methoxy substituted dibromodiazene scaffold (4) as shown in Scheme 3. The bis-para-bromo substituents were exchanged for silyl-protected amines using LiHMDS, Pd_{2}(dba)_{3} and P(t-bu)_{3}. The silyl protecting groups were subsequently removed with HCl. The bis-para-amino substituted tetra-ortho-methoxy diazene (7) was then coupled to the triethylammoniumacetyl chloride (6) with DIPEA in DMF to yield the desired product TOM-QAQ (8).

The synthesis of TMP-QAQ (11), depicted in Scheme 4 diverged from TOM-QAQ at the tetra-ortho-methoxy substituted dibromodiazene scaffold (4). The Boc-protected bis-para-piperazine rings were installed using Buchwald-Hartwig coupling conditions with a Pd_{2}(dba)_{3} catalyst and RuPhos ligand to yield the intermediate 9. Subsequent deprotection of the Boc groups with a solution of 10% TFA in DCM generated free amino groups on the piperazine rings of 10. This product was coupled without purification to triethylammoniumacetyl chloride (6) under basic potassium carbonate in DMF to yield the product TMP-QAQ (11).

Once both analogues, TOM-QAQ and TMP-QAQ, were synthesized, the spectral properties of the two compounds were analyzed to determine optimal wavelengths for photoswitching the compounds for in vivo control of a voltage-gated potassium channel.

TOM-QAQ has a structure similar to that of azobenzenes synthesized by Woolley and colleagues in 2011 except the bis-para-acetamido is replaced with the bis-para-quaternary ammonium-amido to act as an ion channel pore blocker (Figure 9). The bis-para-acetamido TOM derivative was found to undergo robust photoswitching with alternating green light (530 nm) for trans-to-cis isomerization and blue light (455 nm) to return to the more stable trans isomer. The n-π transitions of the trans and cis isomers were separated by 35 nm in DMSO which is large considering these transitions are often overlapping. This permitted efficient photoswitching between the two isomers. Due to the structural similarities of para-acetamido-TOM and
TOM-QAQ, we hoped that the observed separation of the n-π* transitions would be similar for TOM-QAQ.

**Figure 9.** Structures of the para-acetamido-TOM (left) which was previously synthesized and characterized by Woolley and colleagues\(^\text{10}\) as well as TOM-QAQ (right) the open-channel blocking variant described herein.

The photoswitching of TOM-QAQ was analyzed in aqueous buffer under physiological pH to mimic the conditions which would be used for *in vivo* testing. The photoswitching of TOM-QAQ is presented in Figure 10. For para-acetamido-TOM, Woolley and colleagues reported that 530 nm green light promoted isomerization towards the *cis* isomer and 460 nm blue light for isomerization back to *trans*.\(^\text{10}\) A series of wavelengths were tested with TOM-QAQ to determine which wavelengths of light would promote the most robust photoswitching. In Figure 10, 450 nm light was used to promote the *trans* isomer and then various lights of 537 nm, 591 nm, 617 nm and 365 nm were used to enhance the fraction of the *cis* isomer to reach a steady state spectrum. Different lengths of irradiation were necessary to reach the steady state. In Figure 10b, the last irradiation with 537 nm light for an additional 2 min did not change the observed spectrum which suggested that it had reached the steady state where further irradiation would not change the ratio of the isomers present. Despite the shift in the n-π* bands for the *trans* and *cis* isomers of para-acetamido-TOM previously seen by Woolley and colleagues, the bands for TOM-QAQ appeared to have significant spectral overlap since the UV-Vis spectra after irradiation with the various wavelengths of light to promote isomerization between *cis* and *trans* had similar maximal wavelengths of absorbance. The UV-Vis spectral data was collected in aqueous buffer instead of DMSO, as was used for para-acetamido-TOM, and therefore the solvent could be affecting the observed separation of the transitions. It was seen that irradiation with green, amber, red and UV light led to a photostationary state with an enhanced fraction of the *cis* isomer (Figure 10). Irradiation with blue light isomerized TOM-QAQ towards the more stable *trans* isomer. The amber LED was the most effective at isomerizing the compound towards the *cis*
isomer. The n-π* band was observed to undergo a slight hypsochromic shift, however, not as large as the shift reported by Woolley and colleagues for the para-acetamido-TOM in DMSO.\textsuperscript{10} Although spectral overlap was likely limiting efficient switching from occurring, the ability of red light to affect some photoisomerization was promising since the ability of longer wavelength light to penetrate into tissues is significantly increased (Figure 10d). The absorption spectra after irradiation with different wavelengths of light are compiled in Figure 10f. The largest amount of isomerization was observed using 450 nm light to promote the trans isomer and 591 nm light to enhance the fraction of the cis isomer.

**Figure 10.** (a) Effects of irradiation and thermal relaxation on the isomeric state of TOM-QAQ (8). UV-Vis spectra of TOM-QAQ after irradiation with blue light (450 nm) which produces the
trans isomer and (b) green light (537 nm), (c) amber light (591 nm), (d) red light (617 nm) and (e) UV light (365 nm) which enhances the fraction of the cis isomer. (f) Summary of the observed spectra of TOM-QAQ after irradiation with the various wavelengths.

TMP-QAQ bears the TOM substitution pattern with bis-para-piperazine rings linking to the quaternary ammonium. TMP-QAQ is similar to compounds previously studied by Woolley and colleagues in 2013 except the quaternary ammonium was added to allow open-channel blocking of the photochromic ligand (acetamido-TMP, Figure 11). Acetamido-TMP, when cross-linked to a peptide, underwent photoswitching in whole blood demonstrating its use for in vivo biological studies.

Figure 11. Structures of acetamido-TMP (left) which was synthesized and characterized previously by Woolley and colleagues and TMP-QAQ (right) the open-channel blocking variant described herein.

Due to the increased ability of the bis-para-amino substituents to donate electron density into the azobenzene ring, the azo group of TMP-QAQ is able to undergo protonation to form the azonium species (Figure 12a). The combination of the electron rich conjugated system and the proximal H-bonding interactions with the ortho-methoxy substituents allows protonation to occur at a more basic pH than is common for unsubstituted azobenzenes. The $\lambda_{\text{max}}$ of trans TMP-QAQ drastically red-shifts from 390 nm and 460 nm for the neutral trans form to 570 nm for the trans azonium. Woolley and colleagues previously reported that the while the neutral trans form is twisted and non-planar, the trans azonium form is planar which facilitates resonance stabilization and intramolecular H-bond stabilization of the protonated species. The UV-Vis spectra of TMP-QAQ as a function of pH were measured (Figure 12b). The azonium ion was produced as the solution was acidified from a pH of 10.3 until a maximum amount of azonium formed at a pH of 4.5. The absorption spectrum of TMP-QAQ at a neutral pH of 7.4 is highlighted by the black line. Some azonium was present at this pH which greatly red-shifted the absorption
spectrum compared to the neutral azobenzene; this is ideal for long wavelength photoswitching under physiological conditions. The absorbance at the $\lambda_{\text{max}}$ of the trans azonium form is plotted as a function of the pH (Figure 12c). The arrow indicates the apparent pK$_a$, which was determined to be 6.8 using HypSpec. This is comparable to the apparent pK$_a$ of 7.2 previously measured for acetamido-TMP conjugated to a short peptide sequence.\textsuperscript{1} TMP-QAQ having a pK$_a$ near neutral pH is ideal such that some of the trans azonium form is present under physiological conditions and therefore long wavelengths of light can be used for photoswitching \textit{in vivo}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure12}
\caption{(a) Protonation of the \textit{trans} isomer of TMP-QAQ (11) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the \textit{trans} form of TMP-QAQ in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the $\lambda_{\text{max}}$ (570 nm). The apparent pK$_a$ of the azonium was determined to be 6.8, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene (A) and the azonium (AH$^+$), as calculated by HypSpec.}
\end{figure}

Comparing the observed spectral properties of TOM-QAQ and TMP-QAQ, it was observed that increasing the donating ability of the \textit{para}-substituents from the bis-\textit{para}-amide to the bis-\textit{para}-amine, caused a red-shift of the $\lambda_{\text{max}}$ of both the $\pi-\pi^*$ and the n-$\pi^*$ transitions. The observed $\lambda_{\text{max}}$ for trans TOM-QAQ occurred at 358 nm for the $\pi-\pi^*$ transition and at 455 nm for the n-$\pi^*$ transitions.
transition. In the neutral form of trans TMP-QAQ, the observed $\lambda_{\text{max}}$ occurred at 390 nm for the $\pi-\pi^*$ transition and at 460 nm for the $n-\pi^*$ transition, both which were slightly red-shifted compared to TOM-QAQ. The ability of TMP-QAQ to undergo protonation under physiological conditions to form the azonium caused a large bathochromic in the maximum wavelength of absorbance to 570 nm which increases its ability to undergo longer wavelength photoswitching.

The photoswitching of TMP-QAQ was characterized to determine whether it could undergo robust photoswitching to be used as an open-channel blocker in vivo. Azonium ions usually have a thermal relaxation half-life ($\tau_{1/2}$) on the order of microseconds; however, Woolley and colleagues have previously reported thermal back reactions of seconds which was far slower than anticipated rates. Two factors were found to contribute to the slower thermal relaxation: the tetra-ortho substitution pattern appeared to affect the thermal barrier and the deprotonation of the azonium to the neutral cis isomer which accompanied isomerization lead to slower relaxation.

To measure the photoswitching of TMP-QAQ, the setup depicted in Figure 13a was used with a green 537 nm LED as the source and a photomultiplier tube as the detector. A red 617 nm LED was used to isomerize TMP-QAQ. The filters were used to keep the wavelength range of the light source narrow (before the sample cuvette) and to prevent scattering from the isomerizing LED from reaching the detector (after the sample cuvette). Figure 13b shows the photoisomerization of TMP-QAQ. After turning off irradiation with the 617 nm LED, the dark-state spectrum recovered monoexponentially and the $\tau_{1/2}$ of the cis isomer was measured to be 19 s. Multiple photoswitching cycles were repeated over the course of 10 min during which no apparent photobleaching was observed. (Figure 13c).
Figure 13. (a) Setup to measure the photoswitching of TMP-QAQ (pH=7.2). A green 537 nm LED was used as the source. One filter was placed before the sample cuvette to maintain a narrow source beam and a second was placed after the sample cuvette to block scattering from the red 617 nm LED from reaching the detector. (b) Photoswitching of TMP-QAQ. The red bar indicates the photoisomerization under 617 nm irradiation followed by dark-state thermal relaxation (black bar). (c) Multiple photoswitching cycles over 10 min showed no evidence of photobleaching.

The light source was then changed to a blue 420 nm LED to see whether the measuring wavelength would affect the observed half-life (Figure 14a). After irradiation with the 617 nm LED was stopped, the dark-state spectrum recovered thermally (Figure 14b). A monoexponential fit determined the half-life to be 30 s. The discrepancy between the observed half-life of the cis isomer using the 537 nm and 420 nm measuring beam can be potentially explained by a few reasons. The signal-to-noise was significantly better using the 420 nm source than the 537 nm source (Figure 13b vs. Figure 14b). Additionally, there may be a larger difference between the
absorption spectra of the *cis* versus the *trans* isomers at the lower wavelength of 420 nm compared to at 537 nm which would increase the observed signal. The green 537 nm measuring beam may also be causing partial isomerization of TMP-QAQ to *cis*, despite the low intensity of the light source, since the *trans* isomer of TMP-QAQ is strongly absorbing at this wavelength. The ability of TMP-QAQ to be photo-controlled using long-wavelength light at a physiological pH makes it an interesting compound for *in vivo* control of voltage-gated potassium channels.

![Diagram](image)

**Figure 14.** (a) Setup to measure the photoswitching of TMP-QAQ (pH=7.2). A blue 420 nm LED was used as the source. One filter was placed before the sample cuvette to maintain a narrow source beam and a second was placed after the sample cuvette to block scattering from the red 617 nm LED from reaching the detector. (b) Photoswitching of TMP-QAQ. The red bar indicates the photoisomerization under 617 nm irradiation followed by dark-state thermal relaxation (black bar).

In order to assess the ability of TOM-QAQ and TMP-QAQ to silence neuronal excitability in a light-dependent manner, they were tested against an exogenously expressed voltage-gated potassium channel (Kv2.1) in HEK-293 cells. These experiments were run in collaboration with the Isacoff lab at the University of California Berkeley. QAQ, which has previously been shown to confer light sensitivity on voltage-gated potassium channels,\(^\text{13}\) was used as a control to ensure
the channel was being properly expressed. Trans-to-cis isomerization of QAQ occurs under 380 nm UV light. Thermal relaxation in the dark, or irradiation with green light, isomerizes it back to the more stable trans isomer. Some degree of photo-control of the Kv2.1 current by QAQ during depolarizing pulses from -60 mV to +40 mV was demonstrated with UV/green light (Figure 15a) and UV/dark (Figure 15b). When the cell was either under green light irradiation or in the dark, lower currents were produced than under UV light irradiation which is consistent with expected trans-blocking and cis-unblocking of QAQ.13 The shape of the current after the depolarizing pulse, however, was different than that observed by Kramer et al. After depolarization and opening of the expressed Kv channels, we observe an initial decrease in the current followed by an increase until a plateau is reached. In previous studies, an initial increase in the current was observed after depolarization when the channels open followed by a slower decrease in current due to channel inactivation. Further work needs to be done to determine the source of this difference and whether these trace shapes are within the range of expected for Kv2.1 channels. Photoregulation of the voltage-gated potassium channel by QAQ is shown in Figure 15c for a series of depolarizing pulses. The average current at +40 mV was plotted versus the sweep number where the irradiating light source is indicated by the coloured bars. Irradiating with 560 nm light or periods of dark lowered the observed current as was expected for the trans-blocking form. Irradiating with 380 nm light increased the current as it promoted QAQ isomerization to the cis-unblocking form and therefore unblocked the pore. Kramer and colleagues previously reported that QAQ was able to reversibly block Kv2.1 channel currents.13 However, the differences in the observed currents in the trans versus cis forms of QAQ previously reported were far larger than we observed. They observed a difference of 0.8 nA between the currents in the cis unblocking state and the trans blocking state. We observed differences in current between the trans and cis states of only 0.2 nA, four fold smaller than expected current changes. Therefore, while some photoregulation of the current with QAQ was observed, the system is not optimal for testing new compounds since both the shape of the current traces differed from results previously reported and the amount of current regulation observed was four-fold smaller for the control compound. When Trauner and colleagues added the ortho-substituents to QAQ, they observed smaller regulation of the channel currents which was potentially due to steric bulk inhibiting the ability of the compounds to block in the inner vestibule of the voltage-gated potassium channel as effectively as unsubstituted QAQ.14 Due to
the added steric bulk of the tetra-ortho-methoxy substituents of TOM-QAQ and TMP-QAQ, they may have a decreased efficacy as Kv2.1 channel blockers. Therefore, the small changes in current observed for QAQ photo-control is concerning since changes in the current would likely be smaller for the red-shifted analogues.

After testing QAQ as a control to ensure that photoregulation of the exogenously expressed Kv2.1 channels was observed, we ran preliminary testing on TOM-QAQ. As seen in Figure 10, the trans-to-cis isomerization of TOM-QAQ can be promoted using 537 nm, 591 nm, 617 nm or 365 nm light. The isomerization towards trans occurs either slowly through thermal relaxation or via irradiation with 450 nm blue light. Due to absorption spectral overlap of the isomers, we encountered difficulty in getting complete photoswitching which is necessary for in vivo testing to observe the largest possible changes in the current between the two isomers. The current of the HEK-293 cells with intracellular TOM-QAQ application during depolarizing pulses
from -60 mV to +40 mV is shown in Figure 16. Only switching between 580 nm irradiation and periods of dark or 380 nm irradiation and periods of dark were expected to elicit a response based on the wavelengths which were found to be able to photo-control TOM-QAQ. Photo-control of the K\textsubscript{\text{V}	extsubscript{2.1}} channel current was not observed during irradiation with either 580 nm or 380 nm light. Since TOM-QAQ undergoes slow thermal relaxation, the periods of dark may not have been adequately long for relaxation to the trans form. A better experiment would have been to alternate between 530 nm light, or even 600 nm red light, for trans-to-cis isomerization followed by irradiation with 450 nm to photoisomerize back to the more stable trans isomer. It is unclear whether the lack of observed photo-control is due to inability of the compound to block the ion channels, or poor selection of the wavelengths used to photo-control TOM-QAQ during our preliminary patch clamp experiments. Further testing is necessary to validate whether TOM-QAQ could modulate voltage-gated potassium channel currents.

**Figure 16.** Attempted photo-control of K\textsubscript{\text{V}	extsubscript{2.1}} current in HEK-293 cells using intracellular TOM-QAQ (400 \textmu M, pH=7.4). The average current (nA) at +40 mV under irradiation with different wavelengths of light of each depolarizing pulse from -60 mV to +40 mV is shown.

Preliminary testing of internally presented TMP-QAQ was run against the K\textsubscript{\text{V}	extsubscript{2.1}} channel expressed exogenously in HEK-293 cells. TMP-QAQ undergoes trans-to-cis photoswitching with long wavelength 617 nm light and quickly thermally relaxes back to the more stable trans isomer in the dark. The thermal half-life of the cis isomer was determined to be 20-30 s at a neutral pH. The presence of the azonium ion red-shifts the absorption spectrum of TMP-QAQ significantly which allows photo-control of the compound with longer wavelength light. The apparent pK\textsubscript{a} of TMP-QAQ was determined to be 6.8; therefore under physiological pH far more of the neutral azobenzene than the azonium ion is present. Acidification of the internal solution would increase the amount of azonium to promote long-wavelength photo-control of TMP-QAQ. Therefore, the internal solution used with TMP-QAQ was adjusted to a pH of 5.9 for
intracellular application. A preliminary patch clamp experiment using TMP-QAQ for photoregulation of the Kv2.1 channel is depicted in Figure 17. The irradiating light was alternated between a series of wavelengths from 400 nm to 625 nm and periods of dark to see if any wavelength light within that range would be able isomerize TMP-QAQ and alter its ability to block Kv2.1 channel currents. The current was observed to remain steady, aside from noise, throughout the light source changes. There were a few limitations with the experiment which could affect the ability to see photo-control of the ion channel with TMP-QAQ. Firstly, the photo-control of QAQ lead to four-fold smaller changes in current than previously reported and therefore our system is not optimal to see small changes in current. Secondly, testing TMP-QAQ at a pH of 5.9 means that there is a significantly larger proportion of azonium present; however, the relaxation rate would also be predicted to be faster which would make it difficult to maintain a significant fraction of the cis isomer without a powerful light source. Woolley and colleagues found that in the geometry of the cis isomer, the methoxy substituents are too far from the proton on the azo moiety to form an effective H-bond and therefore the pKₐ of the cis isomer is lower than the trans isomer.¹ When the trans azonium undergoes photoisomerization near its pKₐ, the isomerization is coupled to proton loss and generates the neutral cis form. This neutral cis form would undergo slower thermal relaxation than the cis azonium due to an increase in double bond character of the diazene.¹² Woolley and colleagues estimated that the cis azonium had a pKₐ approximately 1.5 units lower than the trans azonium.¹ Therefore, for TMP-QAQ, the decrease in pH to 5.9 for the in vivo study would be closer to the expected pKₐ of the cis azonium of approximately 5.3 and therefore the thermal relaxation rate may be too fast to form a significant proportion of the cis isomer to unblock the ion channel currents. Another limitation of this study was the inability to turn the light source completely off. The ‘dark’ indicated was actually dim 680 nm light. For studying compounds such as QAQ and TOM-QAQ, dim and long wavelength light would not cause photoisomerization since their absorption spectra do not extend far into the red region of the spectrum. However, the tail of the absorbance band for the trans azonium of TMP-QAQ extends to 700 nm so it is not inconceivable that light of this wavelength could be causing some isomerization towards the cis isomer. This would decrease the change in the current observed between the cis unblocking and trans blocking states since some cis isomer would be constantly present. In future studies, the ability to turn on and off the light is crucial instead of using long wavelength light as an ‘off’ due to the ability of TMP-QAQ to absorb at
long wavelengths in the azonium form. Therefore, it remains unclear whether the ion channel blocker TMP-QAQ is unable to block the channels or whether the experimental limitations made it such that no photo-control of the current was observed. Additionally, the acidification of the internal solution to a pH of 5.9 is not ideal since the ion channel blockers need to function under physiological conditions for in vivo studies and therefore future testing should be conducted at a pH of 7.4. Thus, further testing is necessary to validate whether TMP-QAQ could function as a long wavelength photo-controlled ion channel blocker.

![Figure 17](image.png)

**Figure 17.** Attempted photo-control of Kv2.1 current in HEK-293 cells using intracellular TMP-QAQ (100 μM, pH=5.9). The average current (nA) at +40 mV under irradiation with different wavelengths of light of each depolarizing pulse from -60 mV to +40 mV is shown.

### 2.3 Conclusions

Two red-shifted open-channel blockers, TOM-QAQ and TMP-QAQ, for the voltage-gated potassium channel were synthesized, the photoswitching was characterized and preliminary tests against the exogenously expressed voltage-gated Kv2.1 channel in HEK-293 cells were run. Previously, QAQ was shown to be able to photo-control Na⁺, Ca²⁺ and K⁺ channels in vivo. However, light in the UV range was necessary to permit trans-to-cis isomerization. UV light is both damaging to cells and poorly penetrates into tissue which limits the utility of QAQ for photo-control of biological systems. While QAQ was demonstrated to be able to photoregulate the response to pain in nociceptors in the eyes of rats, it would be interesting to study nociceptors outside of the retina. To permit effective tissue penetration, the photoswitch needs to absorb far red or near-infrared light. We made two red-shifted ion channel blockers, TOM-QAQ and TMP-
QAQ, which utilized substitution patterns previously developed by the Woolley lab for red-shifting the absorption spectra to permit longer wavelength photo-control for *in vivo* applications. TOM-QAQ utilized the tetra-*ortho*-methoxy substitution pattern in conjunction with the bis-*para*-amide linker to the quaternary ammonium to mimic QAQ except with the added *ortho*-methoxy substitutions for a bathochromically-shifted absorption spectrum. This compound was able to undergo photoswitching with longer wavelength light than QAQ; green light (537 nm), amber light (591 nm) and even red light (617 nm) enhanced the fraction of the *cis* isomer. However; there was significant spectral overlap of the absorption spectra of the *trans* and *cis* isomers and therefore the photoisomerization observed was not complete. TMP-QAQ bore both the tetra-*ortho*-methoxy substitution pattern in addition to bis-*para*-piperazine linkers between the azobenzene core and the quaternary ammonium pore blockers. The bis-*para*-amino in place of the bis-*para*-amido is far more electron donating and greatly red-shifts the absorption spectrum, as previously shown by Woolley and colleagues. The increase in electron density of the azobenzene ring system in conjunction with H-bonding to a proximal *ortho*-methoxy substituent leads to formation of the TMP-QAQ azonium ion. The azonium form has a red-shifted absorption spectrum which has a λ<sub>max</sub> of 570 nm and a tail which extends out past 700 nm. The apparent p<sub>K</sub><sub>a</sub> of the azonium ion was determined to be 6.8 and therefore some azonium ion is present under physiological conditions. At a neutral pH, TMP-QAQ underwent photoisomerization to *cis* using 617 nm red light and quickly thermally relaxed back to the *trans* isomer with a half-life of 20-30 s. This fast relaxation of TMP-QAQ back to *trans* is ideal to allow efficient photo-control of an ion channel using only long wavelength light to yield the *cis*-unblocking form and then thermal relaxation to relax to the *trans*-blocking form. The fast thermal relaxation means a second, and likely shorter, wavelength of light is not necessary to isomerize back to the more stable *trans* isomer. Preliminary *in vivo* testing of internally presented TOM-QAQ and TMP-QAQ were carried out against the K<sub>V</sub>2.1 channel in HEK-293 cells. While neither compound showed the ability to photo-regulate this channel in the initial studies, further work is required to determine if they could be effective open-channel blockers. As was described by Trauner and colleagues, some substitutions to the *ortho*-positions on the QAQ scaffold permitted reversible photo-control of Shaker-IR K<sup>+</sup> current. However, the changes in the current were smaller than those observed with unsubstituted QAQ and the necessary concentrations of channel blocker to achieve photo-regulation of the channels were higher. It
has been hypothesized that the inner vestibule of voltage-gated potassium channels is not tolerant to larger substituents which is potentially why a decrease in efficacy was seen when Trauner and colleagues added symmetrical *ortho*-methoxy groups at the 2,2’ positions on QAQ. The increased steric size of TOM-QAQ and TMP-QAQ, since they bear the tetra-*ortho*-methoxy substitution pattern, compared to the unsubstituted QAQ may not be well tolerated by the inner vestibule of the K\textsubscript{V}2.1 ion channel. Fortunately, quaternary ammoniums block an array of ion channels; thus, further testing of TOM-QAQ and TMP-QAQ against sodium, calcium and other potassium channels would also be interesting since the other channels may have different steric accommodations. Additionally, there is an external quaternary ammonium binding site on voltage-gated potassium channels on the outside of the cell membrane which is less sterically hindered than the inner channel binding site.\textsuperscript{15} Therefore, external application of TOM-QAQ and TMP-QAQ would have the potential to modulate ion channel currents. For TMP-QAQ, the addition of bis-*para*-piperazine linkers also increases the flexibility of the compound which could minimize the change in the geometry due to isomerization. This could reduce the ability of TMP-QAQ isomerization to modulate the ion channel currents since the sterics of the *trans* versus *cis* isomers are hypothesized to affect the ability of the quaternary ammonium to bind and block the channel within the inner vestibule. It would be interesting to synthesize the unsubstituted version (P-QAQ, Figure 18) for comparison. While this compound would be less red-shifted than the tetra-*ortho*-methoxy substituted TMP-QAQ, it would test whether the ion channel is tolerant to the addition of the piperazine linkers and if current photo-control would be retained. For external blocking of ion channels with tethered quaternary ammonium photochromic ligands, the length of the linker seems to be critical.\textsuperscript{16} This could therefore be an interesting route to explore with compounds similar to TMP-QAQ. Lastly, if the tetra-*ortho*-methoxy substitution pattern is accepted by the channel then it would be interesting to utilize our new red-shifted DOM substitution pattern, which will be discussed in further detail in Chapter 3. It would be straight-forward to make the DOM versions of TOM-QAQ and TMP-QAQ (DOM-QAQ and DMP-QAQ, Figure 18) for further red-shifted absorption spectra.
2.4 References


Chapter 3
A Red-Shifted Azobenzene Substitution Pattern

3.1 Methods

General

All commercial materials (solvents, reagents and substrates) were used as received. SiliaFlash P60 silica gel of particle size 40-63 μm was used for column chromatography (Silicycle Inc.) 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. A yield is not reported for some of the compounds as it was not feasible to purify the entire crude product by HPLC and only enough was purified to collect spectral data and characterize the absorption spectral properties. One-dimensional \(^1\)H and \(^13\)C NMR spectra were recorded on the Varian UnityPlus 500 MHz spectrometer. The chemical shifts of the spectra are reported in parts per million and the spectra were referenced to the residual NMR solvent signals. For \(^1\)H NMR: chloroform-\(d\) (7.26 ppm), methanol-\(d_4\) (3.31 ppm), DMSO-\(d_6\) (2.50 ppm), deuterium oxide (4.79 ppm) and acetone-\(d_6\) (2.05 ppm). For \(^13\)C NMR: chloroform-\(d\) (77.16 ppm), methanol-\(d_4\) (49.00 ppm), DMSO-\(d_6\) (39.52 ppm) and acetone-\(d_6\) (29.84, 206.26 ppm). \(^1\)H NMR spectral characterization is reported as follows: chemical shift (multiplicity, coupling constant, integration, assignment, isomer (if necessary)). The adjacent quadrupolar \(^{14}\)N nuclei may be contributing to missing sp\(^2\) hybridized \(^{13}\)C resonances. 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.
Scheme 5. Buchwald-Hartwig coupling of diBr-TOM (4) with various amines to access TOM-substituted compounds 12 to 14.°

To an oven-dried pressure tube, cooled under nitrogen gas, was added 4 (20 mg, 0.04 mmol), 4-methoxypiperidine (35 mg, 0.30 mmol), RuPhos (2.3 mg, cat.), Pd₂(dba)₃ (2.3 mg, cat.), Cs₂CO₃ (98 mg, 0.30 mmol) and anhydrous toluene (3 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 18 h. The reaction was cooled to room temperature. The crude material was extracted with EtOAc, washed with brine, dried with Na₂SO₄ and concentrated under vacuum. Column chromatography (Methanol: DCM (1:9)) yielded the semi-pure product. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid. ¹H NMR (500 MHz, Deuterium Oxide) δ 1.47 (m, 4H, CH₄), 1.92 (s, 4H, CH₂), 2.94 (m, 4H, CH₂), 3.26 (s, 6H, OCH₃), 3.45 (m, 2H, CH), 3.55 (m, 4H, CH₂), 3.73 (s, 12H, OCH₃), 6.19 (s, 4H, Ar). ¹³C NMR (126 MHz, Deuterium Oxide) δ 29.50, 46.34, 54.79, 55.82, 76.08, 150.52. ESI-Q-TOF: m/z calc. for C₂₈H₄₀N₄O₆: 529.3021 [M+H]⁺; found 529.3016.

°Conditions and reagents. (a) select R-H reagent, Pd₂(dba)₃, RuPhos, Cs₂CO₃, toluene, 100 °C, 18-26 h, yield 40-75%.
1,2-bis(2,6-dimethoxy-4-(3-methoxypyrrolidin-1-yl)phenyl)diazene (13)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 4 (20 mg, 0.04 mmol), 3-methoxypyrrolidine·HCl (30 mg, 0.30 mmol), RuPhos (2.5 mg, cat.), Pd$_2$(dba)$_3$ (2.5 mg, cat.), Cs$_2$CO$_3$ (98 mg, 0.30 mmol) and anhydrous toluene (2.5 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 26 h. The crude product was filtered through celite and then the solvent was removed in vacuo. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid (yield 75% by HPLC). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 2.07 (m, 4H, CH$_2$), 3.24 (s, 0.8H, OCH$_3$, cis), 3.27 (s, 6H, OCH$_3$, trans), 3.36 (m, 6H, CH/CH$_2$), 3.48 (m, 2H, CH$_2$), 3.66 (s, 1.44H, OCH$_3$, cis), 3.69 (s, 12H, OCH$_3$, trans), 4.08 (m, 2H, CH$_2$), 5.74 (s,0.3H, Ar, cis), 5.82 (s, 4H, Ar, trans). Ratio trans:cis (92:8). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 30.64, 46.09, 53.30, 55.24, 56.48, 89.85, 90.99, 158.11, 158.35. Note: TFA resonances observed at δ 117.50 (q), 161.58 and chloroform at δ 79.61. ESI-Q-TOF: m/z calc. for C$_{26}$H$_{36}$N$_4$O$_6$: 501.2708 [M+H]$^+$; found 501.2703.

1,2-bis(2,6-dimethoxy-4-(3-methoxazetidin-1-yl)phenyl)diazene (14)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 4 (10 mg, 0.02 mmol), 3-methoxazetidine·HCl (16.5 mg, 0.12 mmol), RuPhos (2 mg, cat.), Pd$_2$(dba)$_3$ (2 mg, cat.), Cs$_2$CO$_3$ (40 mg, 0.12 mmol) and anhydrous toluene (2.5 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 21 h. The crude product was filtered through celite and then the solvent was removed in vacuo. Column chromatography (Methanol: DCM (1:19)) afforded the semi-pure product. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a purple solid (yield 40% by HPLC). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 3.24 (s, 6H, OCH$_3$), 3.65 (s, 12H, OCH$_3$), 3.67 (m, 4H, CH$_2$), 4.11 (m, 4H, CH$_2$), 4.32 (m, 2H, CH), 5.71 (s, 4H, Ar). $^{13}$C
NMR (126 MHz, DMSO-\textit{d}_6) \delta 55.40, 55.89, 58.43, 68.98, 88.54, 153.56. Note: DCM signal observed at 54.91. ESI-Q-TOF: \textit{m/z} calc. for C_{24}H_{32}N_{4}O_{6}: 473.2395 [M+H]^+; found 473.2402.

Scheme 6. Buchwald-Hartwig coupling of diBr-DOM (15) with various amines to access DOM-substituted compounds 16 to 19.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound #</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>6-OMe-DOM</td>
<td>5-OMe-DOM</td>
<td>4-OMe-DOM</td>
<td>diEt-DOM</td>
</tr>
<tr>
<td>R-H group</td>
<td>\includegraphics[width=0.1\textwidth]{16.png}</td>
<td>\includegraphics[width=0.1\textwidth]{17.png}</td>
<td>\includegraphics[width=0.1\textwidth]{18.png}</td>
<td>\includegraphics[width=0.1\textwidth]{19.png}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions and reagents. (a) select R-H reagent, Pd\textsubscript{2}(dba)\textsubscript{3}, RuPhos, Cs\textsubscript{2}CO\textsubscript{3}, toluene, 100 °C, 16-26 h, yield \textasciitilde50%.

1,2-bis(6-methoxy-8-(4-methoxypiperidin-1-yl)-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)diazene (16)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 15 (20 mg, 0.04 mmol), 4-methoxypiperidine (35 mg, 0.30 mmol), RuPhos (2.3 mg, cat.), Pd\textsubscript{2}(dba)\textsubscript{3} (2.3 mg, cat.), Cs\textsubscript{2}CO\textsubscript{3} (98 mg, 0.30 mmol) and anhydrous toluene (6 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 24 h. The reaction was cooled to room temperature. The crude material was extracted with EtOAc, washed with brine, dried with Na\textsubscript{2}SO\textsubscript{4} and then dried under vacuum. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid. \textsuperscript{1}H NMR (500 MHz, Chloroform-\textit{d}) \delta 1.80 (m, 4H, CH\textsubscript{2}), 2.03 (m, 4H, CH\textsubscript{2}), 2.85 (m, 4H, CH\textsubscript{2}), 3.37 (m, 6H, CH\textsubscript{2}/CH\textsubscript{3}), 3.39 (s, 6H, OCH\textsubscript{3}), 3.81 (s, 6H, OCH\textsubscript{3}), 4.32 (s, 8H, CH\textsubscript{2}), 6.24 (s, 2H, Ar). \textsuperscript{13}C NMR (126 MHz, Chloroform-\textit{d}) \delta 30.92, 48.26, 55.60, 57.25, 63.60, 64.57, 96.36, 130.81, 138.28, 142.52, 147.13. ESI-Q-TOF: \textit{m/z} calc. for C\textsubscript{30}H\textsubscript{40}N\textsubscript{4}O\textsubscript{8}: 585.2919 [M+H]^+; found 585.2911.
1,2-bis(6-methoxy-8-(3-methoxypyrrolidin-1-yl)-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)diazene (17)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 15 (20 mg, 0.04 mmol), 3-methoxypyrrolidine-HCl (33 mg, 0.24 mmol), RuPhos (2.3 mg, cat.), Pd$_2$(dba)$_3$ (2.3 mg, cat.), Cs$_2$CO$_3$ (98 mg, 0.30 mmol) and anhydrous toluene (2.5 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 26 h. The crude product was filtered through celite and then the solvent was removed in vacuo. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid (yield 43% by HPLC). $^1$H NMR (500 MHz, Chloroform-$d$) δ 2.06 (m, 4H, CH$_2$), 3.36 (s, 6H, OCH$_3$), 3.46 (m, 4H, CH$_2$), 3.53 (m, 2H, CH$_2$), 3.72 (m, 2H, CH$_2$), 3.84 (s, 6H, OCH$_3$), 4.04 (m, 2H, CH), 4.24 (m, 4H, CH$_2$), 4.36 (m, 4H, CH$_2$), 5.95 (s, 2H, Ar). $^{13}$C NMR (126 MHz, Chloroform-$d$) δ 30.52, 48.27, 55.94, 56.59, 57.03, 63.16, 64.83, 79.85, 92.77, 128.00, 139.63. ESI-Q-TOF: m/z calc. for C$_{23}$H$_{36}$N$_4$O$_8$: 557.2606 [M+H]$^+$; found 557.2596.

1,2-bis(6-methoxy-8-(3-methoxyazetidin-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 15 (10 mg, 0.02 mmol), 3-methoxyazetidine-HCl (16.5 mg, 0.12 mmol), RuPhos (2 mg, cat.), Pd$_2$(dba)$_3$ (2 mg, cat.), Cs$_2$CO$_3$ (40 mg, 0.12 mmol) and anhydrous toluene (2.5 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 20 h. The crude product was filtered through celite and then the solvent was removed in vacuo. Column chromatography (Methanol: DCM (1:19)) afforded the semi-pure product. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid (yield 50% by HPLC). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 3.22 (s, 6H, OCH$_3$), 3.63 (s, 6H, OCH$_3$), 3.69 (m, 4H, CH$_2$), 4.11 (m, 8H, CH$_2$), 4.15 (m, 4H, CH$_2$), 4.22 (m, 2H, CH), 5.70 (s,
2H, Ar). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 55.80, 57.06, 60.65, 63.57, 64.55, 70.16, 91.15, 127.02, 137.55, 141.00, 147.31. ESI-Q-TOF: m/z calc. for C$_{24}$H$_{32}$N$_4$O$_6$: 529.2293 [M+H]$^+$; found 529.2290.

8,8'-(diazene-1,2-diyl)bis(N,N-diethyl-7-methoxy-2,3-dihydrobenzo[b][1,4]dioxin-5-amine) (19)

To an oven-dried pressure tube, cooled under nitrogen gas, was added 15 (20 mg, 0.04 mmol), diethylamine (50 μL, 0.48 mmol), RuPhos (2 mg, cat.), Pd$_2$(dba)$_3$ (2 mg, cat.), Cs$_2$CO$_3$ (230 mg, 0.72 mmol) and anhydrous toluene (3 mL). The tube was sealed with a Teflon stopper and heated to 100 °C for 16 h. The solution was cooled to room temperature and the solvent was removed in vacuo. The crude product was dissolved in DCM, washed with brine, dried over anhydrous Na$_2$SO$_4$ and concentrated under vacuum. Column chromatography (Methanol: DCM (1:19)) afforded the semi-pure product. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid. $^1$H NMR (500 MHz, DMSO-$d_6$) δ 1.02 (t, $J$ = 7.0 Hz, 12H), 3.18 (q, $J$ = 7.0 Hz, 8H), 3.64 (s, 6H), 4.16 (m, 8H), 6.18 (s, 2H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 12.88, 45.46, 57.13, 63.52, 64.42, 97.86, 146.10. ESI-Q-TOF: m/z calc. for C$_{26}$H$_{36}$N$_4$O$_6$: 501.2708 [M+H]$^+$; found 501.2711.

1,2-bis(6-methoxy-8-morpholino-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)diazene (20)

Synthesized by our collaborator Mingxin Dong. Reverse-phase HPLC (Zorbax RX-C8 column with a linear gradient from 5% to 70% ACN/water containing 0.1% trifluoroacetic acid over 25 min) yielded the pure product as a blue solid (2 mg). $^1$H NMR (500 MHz, Chloroform-$d$) δ 3.13 (m, 8H, CH$_2$), 3.61 (s, 1H, OCH$_3$, cis), 3.83 (s, 6H, OCH$_3$, trans), 3.90 (m, 8H, CH$_2$), 4.32 (m, 8H, CH$_2$), 6.01 (s, 0.37 H, Ar, cis), 6.21 (s, 2H, Ar, trans). Ratio trans:cis (85:15). $^{13}$C NMR (126 MHz, Chloroform-$d$) δ 50.87, 57.32, 63.58, 64.59, 66.98, 95.86, 130.79, 138.36, 141.78, 147.23. ESI-Q-TOF: m/z calc. for C$_{26}$H$_{36}$N$_4$O$_8$: 529.2293 [M+H]$^+$; found 529.2290.
Sample preparation for UV-Visible spectroscopy

Solutions were prepared in universal buffer with a mixture of four components (CAPSO, TRIS, MES, sodium acetate, 5 mM each) unless otherwise specified to ensure the pH could be easily adjusted between 2 and 11 by addition of small quantities of concentrated hydrochloric acid or sodium hydroxide. The compounds were dissolved in DMSO and then diluted with buffer with the DMSO percentage in the final solution being 5%.

pH-dependence of absorption spectra

The solutions were prepared with the compounds dissolved in 5% DMSO in 5 mM of universal buffer as previously described. Small quantities of concentrated 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. All spectra were baseline-corrected, assuming zero absorption at 800 nm. HypSpec (version 1.2.31)\(^{1-2}\) was used to calculate the apparent pK\(_a\) for the trans form of each compound using the UV-visible spectral data and the corresponding pH. These data are presented in Figure 24 (12), Figure 25 (13), Figure 26 (14), Figure 27 (16), Figure 28 (20), Figure 29 (17), Figure 34 (18), and Figure 35 (19).

Constants are reported as log(\(\beta\)) by HypSpec. To determine the pK\(_a\), the following equations were used, where A represents the parent neutral azobenzene, \(\beta\) is the acid association constant and \(K_a\) is the acid dissociation constant.

For a single protonation event (azonium formation):

\[
\begin{align*}
A + H^+ \rightleftharpoons AH^+ \\
K_{\alpha 1} = \frac{[AH^+]}{[A][H^+]} = \frac{1}{\beta_1} \\
\log(K_{\alpha 1}) = -\log(\beta_1) \therefore pK_{\alpha 1} = \log(\beta_1)
\end{align*}
\]

For a double protonation event (azonium plus ammonium):

\[
\begin{align*}
\begin{align*}
\end{align*}
\end{align*}
\]
\[
A + H^+ \rightleftharpoons AH^+, \quad A + 2H^+ \rightleftharpoons AH^2^{2+}
\]

\[
AH^2^{2+} \rightleftharpoons AH^+ + H^+
\]

\[
K_{a2} = \frac{[AH^+][H^+]}{[AH^2^{2+}]}
\]

where \([AH^+] = \beta_1[A][H^+], \quad [AH^2^{2+}] = \beta_2[A][H^+]^2\),

therefore \(K_{a2} = \frac{(\beta_1[A][H^+])[H^+]}{\beta_2[A][H^+]^2}, \quad K_{a2} = \frac{\beta_1}{\beta_2}\)

\[
\log(K_{a2}) = \log(\beta_1) - \log(\beta_2)
\]

\[
pK_{a2} = - \log(\beta_1) + \log(\beta_2)
\]

**Time-dependent decrease in azonium ion of 5-OMe-DOM (17)**

The apparent decrease was monitored in several solutions: 5% DMSO in 5 mM universal buffer at pH=7.5 and 5% DMSO in 5 mM sodium phosphate buffer at pH=7.5 to test the effect of the buffer. To test the solubility, the decrease was monitored in 30% DMSO in 5 mM universal buffer at pH=7.5 and 30% MeOH in 5 mM universal buffer at pH=7.7. To test the effect of pH on the decrease, it was run in 7% DMSO in 5 mM universal buffer at pH=5.9 and 12% DMSO in water at pH=8. The pH of the solutions were adjusted using a glass combination micro-electrode (MI-710, Microelectrodes Inc.). UV-Vis spectra were acquired on the PerkinElmer Lambda-35 instrument at 5 min intervals to monitor the apparent decrease in the azonium ion. These data are presented in Figure 30, Figure 31 and Figure 32. Fitting the absorbance as a function of time with a monoexponential decay process gave the observed kinetic constants for the half-lives of the decay. These data are summarized in Table 2.

**Thin-layer chromatography of the apparent decrease of 5-OMe-DOM (17)**

A sample of 5-OMe-DOM (17) was left in a solution of water with 5% DMSO at a pH of 7.5 for 16 h. Thin-layer chromatography in 10% MeOH/DCM was run to compare the product of the decrease to the pure standard. The plate was visualized in ambient light, under UV light irradiation with a Mineralight Lamp (Model UVG-11, 254 nm) and with a KMnO₄ stain. These data are presented in Figure 33.
Computational Methods

DFT and time-dependent DFT (TDDFT) calculations were carried out using Gaussian 09 package.\(^3\) Gauss View 5\(^4\) was used to sketch the initial geometries by manually modifying the optimized structures of 12 in the neutral and protonated forms from our previous study.\(^5\) For simplicity, the piperidino analogues of 12 and 16, pyrrolidino analogues of 13 and 17, azetidino analogues of 18, and diethylamino (19) were used (Figure 19). All structures were optimized in vacuo using B3LYP\(^6\) hybrid functionals and the 6-31++G(d,p) (12, 13) or 6-31+G(d,p) (16, 17, 18, 19) basis set. Frequency calculations were performed at the same level of theory to confirm that the optimization results represented local minima on the energy landscape. An exhaustive conformational search was not carried out. For sketching the protonated forms of 16, 17, 18 and 19, the proton was placed opposite the dioxane ring on the proximal nitrogen of the azo bond (Figure 19d). Avogadro\(^7\)\(^-\)\(^8\) software was used to measure the dihedral angles.

![Diagram of molecules](image)

**Figure 19.** Neutral trans form (a) and azonium trans form (c) of 12’ and 13’ bearing the TOM substitution pattern. Neutral trans form (b) and azonium trans form (d) of 16’, 17’, 18’ and 19’ bearing the DOM substitution pattern.
3.2 Results and Discussion

Azobenzenes bearing the tetra-ortho-methoxy (TOM) substitution pattern in conjunction with bis-para-amino substituents have previously been shown to have desirable red-shifted absorption bands, especially when in the protonated azonium form.\textsuperscript{9} These azobenzenes can be photoswitched from \textit{trans}-to-\textit{cis} with long-wavelength light of 660 nm.\textsuperscript{10} While this is a great improvement compared to the unsubstituted azobenzene core which undergoes \textit{trans}-to-\textit{cis} isomerization using 380 nm UV light, there is a constant desire to further red-shift the core since the optimal tissue penetration for photoswitch use in biological systems occurs in the near-IR.\textsuperscript{11-12} Based on Wizinger and colleagues work on the effects of the distribution of auxochromes, it was predicted that placing a methoxy substituent opposite the methoxy ortho to the diazene bond would further red-shift the absorption spectrum.\textsuperscript{13} Woolley and colleagues made a derivative with this ortho-meta substitution of the methoxy groups in conjunction with the bis-para-piperazine rings and observed that while the absorption spectra of the neutral \textit{trans} isomer and the \textit{trans} azonium were red-shifted, the rate of thermal relaxation was far faster than with TOM substitution (Figure 20).\textsuperscript{10} It was hypothesized that the sterics of the four tetra-ortho-methoxy groups significantly slow the rate of thermal relaxation. Additionally, the apparent pK\textsubscript{a} of the azonium ion decreased from 7.5 for the TOM-substituted to 2.6 for the ortho-meta-methoxy substituted derivative. This decrease in pK\textsubscript{a} was attributed to a twist in the dihedral angle between the azobenzene rings and the para-substituent due to steric hindrance with the meta-methoxy which limited the ability of the bis-para-amines to donate electron density into the ring system. TDDFT calculations showed that the ability of the methoxy group to act as a H-bond acceptor was still optimal so the H-bonding was not contributing to the decreased pK\textsubscript{a}.\textsuperscript{10} Thus, to optimize the substitution pattern to benefit from not only the further red-shifted absorption spectrum, but also the slower rate of thermal relaxation and the higher pK\textsubscript{a}, the dioxane-ortho-methoxy (DOM) substitution pattern was designed (Figure 20).
In the novel DOM substitution pattern, the ortho-meta placement of the methoxy auxochromes was retained, but an additional methoxy was added ortho to the diazene to promote slower thermal relaxation. The two methoxy groups on the same side of the ring were linked into a dioxane-like ring to minimize steric hindrance of the substituents with the bis-para-amino functionality and with each other. Based on TDDFT calculations, steric clash of the meta-methoxy group with the para-amino substituents decreased the ability of the nitrogen lone pairs to donate electron density into the ring causing a decrease in the pK$_a$. Thus, restricting the meta-methoxy in the dioxane ring in the DOM substitution pattern was expected to minimize the steric hindrance with the para-substituents to promote azonium formation under physiological conditions.

The diBr-DOM (15) starting material was synthesized by our collaborator Mingxin Dong at the Beijing Institute of Biotechnology (Figure 21). To study the novel scaffold, we synthesized a series of azobenzenes with different bis-para-amino groups with both the TOM and the DOM-substituted cores for comparison of the properties (Figure 22, Figure 23). Initially Buchwald-Hartwig cross coupling of various amines with diBr-TOM and diBr-DOM was used to access the compounds. For the cross-coupling reactions, Pd$_2$(dba)$_3$ with a RuPhos ligand was used as the catalyst. From each cross-coupling reaction, some unreacted starting material, some of the

Figure 20. TOM, ortho-meta-methoxy and DOM substitution patterns.

Figure 21. Starting materials diBr-TOM (4) and diBr-DOM (15) for red-shifted azobenzenes.
mono-reacted and some of the desired di-reacted product formed and therefore the yields of the products were quite variable (from 40-75%) despite using an excess of the amines. The relative ability of amines of various ring sizes to donate electron density into the azobenzene ring system was studied by making a series of compounds bearing 6-, 5- and 4-membered amine ring substituents para to the diazene bond. The smaller rings were expected to decrease the steric hindrance with the meta-substituent decreasing the dihedral angle for better resonance with the conjugated aromatic system. The bis-para-amine rings were functionalized with a methoxy substituent to increase the water solubility of the compounds for testing the spectral properties under aqueous conditions. The compounds bearing the tetra-ortho-methoxy substitution pattern are depicted in Figure 22 and the compounds bearing the dioxane-ortho-methoxy substitution pattern are depicted in Figure 23.

**Figure 22.** Summary of compounds bearing the TOM substitution pattern.
Once the library of compounds bearing both the TOM and DOM azobenzene substitution patterns were synthesized, their spectral properties were analyzed using UV-Vis spectroscopy. For 6-OMe-TOM (12), the UV-Vis spectra are depicted in Figure 24. The pH dependence of the absorption spectrum of the *trans* form is shown (Figure 24b). At the highest pH, the spectrum represents the neutral *trans* azobenzene and the two absorbance maxima occurred at 458 nm and 395 nm. As the pH was lowered, a species of long wavelength absorption appeared ($\lambda_{\text{max}}$=545 nm), which is assumed to be the singly protonated azonium ion. The tail of the absorbance peak for the azonium form extends out to 710 nm in the near-IR. Under acidic conditions, the absorption spectrum plateaued which is assumed to be the fully protonated 6-OMe-TOM azonium. The absorption spectrum at a neutral pH is indicated by the black line which indicates that a significant amount of the azonium is present under physiological conditions. The maximum absorbance of the azonium at 545 nm is plotted as a function of pH (Figure 24c). The apparent $pK_a$ of the azonium ion was determined to be 7.7 using HypSpec.
which is shown by the vertical arrow. The deconvoluted absorption spectra for the neutral \textit{trans} form (A) and the \textit{trans} azonium (AH\textsuperscript{+}) are shown in Figure 24d.

\textbf{Figure 24.} (a) Protonation of the \textit{trans} form of 6-OMe-TOM (12) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the \textit{trans} form of 12 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maxima (545 nm). The apparent \( pK_a \) of the azonium ion was determined to be 7.7, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene and the azonium, as calculated by HypSpec.

The characterization of the spectral properties of 5-OMe-TOM (13), bearing the bis-\textit{para}-pyrrolidines, is shown in Figure 25. The neutral \textit{trans} azobenzene, which is assumed to be the only form present at the high pH limit, has absorbance maxima of 485 nm and 435 nm (Figure 25b). Acidification of the compound produced the \textit{trans} azonium which had \( \lambda_{\text{max}} \) bands at 600 nm and 560 nm. In the azonium form, the tail of the absorption band extended out to 730 nm in the near-IR. Under physiological conditions, the compound was almost completely in the protonated azonium form since the apparent \( pK_a \) was determined to be 9.1.
**Figure 25.** (a) Protonation of the *trans* form of 5-OMe-TOM (13) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the *trans* form of 13 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maximum (560 nm). The apparent pK<sub>a</sub> of the azonium ion was determined to be 9.1, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene and the azonium, as calculated by HypSpec.

For the TOM derivative coupled to the 4-membered azetidine ring, 4-OMe-TOM (14), the UV-Vis spectra are depicted in Figure 26. Under the most basic conditions, the absorption spectrum is assumed to be the neutral *trans* form which had absorbance maxima occurring at 465 nm and 410 nm (Figure 26b). Acidification produced the azonium ion which has a red-shifted λ<sub>max</sub> of 545 nm. The tail of the absorption band of the azonium ion extends out to 710 nm. The maximal absorbance is plotted as a function of pH (Figure 26c) where the apparent pK<sub>a</sub> of the azonium ion was determined to be 8.3.
Figure 26. (a) Protonation of the *trans* form of 4-OMe-TOM (14) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the *trans* form of 14 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maximum (545 nm). The apparent pKₐ of the azonium ion was determined to be 8.3, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene and the azonium, as calculated by HypSpec.

The spectral properties for the TOM-substituted azobenzenes are tabulated in Table 1. The nature of the bis-para-amines affects the ability of the nitrogen lone pairs to delocalize electron density into the azobenzene ring system.¹⁶ For optimal electron donation, the dihedral angle between the para substituent and the azobenzene ring needs to be minimized so the nitrogen lone pairs can participate fully in the extended conjugation. In both the neutral *trans* form and the *trans* azonium, the absorbance maxima are further red-shifted for 5-OMe-TOM than 6-OMe-TOM or 4-OMe-TOM. The absorbance band for the *trans* azonium is also bathochromically shifted and extends out further into the near-IR. Increasing the electron donation of the bis-para-amino substituents red-shifts the absorption; therefore, the 5-membered pyrrolidine ring is the best electron donor. The predicted dihedral angle for 6-OMe-TOM of 6° and 5-OMe-TOM of 4° supports the observation that the pyrrolidino rings are better able to participate in the extended conjugation. This was the expected result since pyrrolidino rings have smaller bond angles than
piperidino rings and therefore there would have less steric hindrance with the azobenzene rings permitting them to lie closer to planar. It is interesting that the azetidino derivative (4-OMe-TOM), which had a predicted dihedral angle of 2°, was not even more electron donating than 5-OMe-DOM due to an even smaller steric hindrance between the two rings. This could be explained by an increase in ring strain in the 4-membered derivative which would increase the p-character in the N-C bonds. Thus, the nitrogen lone pair would have less p-character and therefore would not be able to participate in resonance to the same extent despite the smaller dihedral angle. This could explain why the predicted maximum wavelength of absorbance of the azonium is 588 nm whereas an absorbance maxima of only 545 nm was observed experimentally. The 5-OMe-TOM derivative was found to have the highest apparent pKₐ of the azonium ion of 9.1, compared to 7.7 for 6-OMe-TOM and 8.3 for 4-OMe-TOM. The increased electron density of the azobenzene ring system increases the ease of the azonium formation and thus it is stable at a higher pH.

Table 1. TDDFT predicted (B3LYP (6-31++G**)) and experimentally observed spectral properties for DOM-substituted azobenzene derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Predicted λ_max (trans neutral form) (nm)</th>
<th>Observed λ_max (trans neutral form) (nm)</th>
<th>Predicted λ_max (trans azonium form) (nm)</th>
<th>Observed λ_max (trans azonium form) (nm)</th>
<th>Maximum λ absorbance tail (nm)</th>
<th>Predicted dihedral angle (°)</th>
<th>Apparent pKₐ of azonium</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OMe-TOM</td>
<td>523</td>
<td>458, 395</td>
<td>545</td>
<td>545</td>
<td>710</td>
<td>6</td>
<td>7.7</td>
</tr>
<tr>
<td>5-OMe-TOM</td>
<td>518</td>
<td>485, 435</td>
<td>544</td>
<td>600, 560</td>
<td>730</td>
<td>4</td>
<td>9.1</td>
</tr>
<tr>
<td>4-OMe-TOM</td>
<td>–</td>
<td>465, 410</td>
<td>588</td>
<td>545</td>
<td>710</td>
<td>2</td>
<td>8.3</td>
</tr>
</tbody>
</table>

For the DOM-substituted azobenzene, the same series of bis-para-amino rings were attached via Buchwald-Hartwig coupling to compare the effect of the ring size on the absorption spectra of the compounds. For 6-OMe-DOM (16), bearing the para-piperidine rings, the spectral data are shown in Figure 27. At the highest pH, the neutral trans form predominates which had absorbance maxima at 458 nm and 378 nm. This appears to be the only form of the compound present under physiological conditions (Figure 27b). Acidification produced the azonium ion, red-shifting the absorbance maximum to 560 nm with the band extending to 720 nm. Further acidification, however, formed the doubly protonated species (azonium plus ammonium) which hypsochromically shifted the λ_max to 500 nm. The apparent pKₐ of the azonium ion was determined to be 5.2 and for the doubly protonated form (azonium plus ammonium) the apparent pKₐ was 3.6. A clear isosbestic point was not observed for the pH titration since more than two species were contributing to the observed UV-Vis spectra. The deconvoluted spectra calculated
by HypSpec for the three species, the neutral trans (A), the azonium (AH⁺) and the azonium plus ammonium (AH₂²⁺), are shown in Figure 27d.

![Diagram](image)

**Figure 27.** (a) Protonation of the trans form of 6-OMe-DOM (16) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the trans form of 16 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). Further decreasing the pH produced the doubly protonated species (azonium plus ammonium), indicated by the dotted grey lines growing to a maximum concentration (dotted red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maximum (560 nm). The pKₐ of the azonium formation was determined to be 5.2 and the pKₐ for the doubly protonated species was 3.6. The solid arrow marks the pKₐ of the azonium ion. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene, the azonium and the doubly protonated species, as calculated by HypSpec.

Comparable to 6-OMe-DOM (16) was the 6-morpho-DOM (20) compound which also had a 6-membered nitrogen-containing ring substituted at the bis-para-positions except it bore morpholine rings in place of the piperidine substituents. The neutral trans isomer, which had maximal absorbance at 470 nm and 371 nm, was the form present under neutral conditions (Figure 28b). Acidification of the neutral form to the azonium caused a bathochromic shift of the absorbance maximum to 569 nm with a tail extending out to 745 nm in the near-IR. The apparent pKₐ of azonium formation was determined to be 4.4, as indicated by the arrow in Figure 28c.
Since there were only two species, neutral *trans* and *trans* azonium, a clear isosbestic point was observed. The spectral properties were similar to those observed for 6-OMe-DOM which was expected since both 6-membered N-containing rings should have similar steric interactions with the azobenzene rings giving similar extents of electron donation.

**Figure 28.** (a) Protonation of the *trans* form of 6-morpho-DOM (20) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the *trans* form of 20 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the maximum wavelength of absorption (569 nm). The apparent pKₐ of the azonium ion was determined to be 4.4, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene and the azonium, as calculated by HypSpec.

For 5-OMe-DOM (17), the spectral data is presented in Figure 29. At the highest pH, the spectrum is assumed to represent the neutral *trans* form. It was found to have absorbance maxima at 486 nm and 440 nm (Figure 29b). As the compound was acidified, the protonation of the diazene caused a red-shift in the maximum wavelength of absorbance to 575 nm with the tail extending out to 775 nm. The absorbance as a function of pH is shown; the apparent pKₐ of the azonium ion was determined to be approximately 7.9 (Figure 29c). The near-neutral apparent pKₐ means that a significant proportion of the azonium ion would be present for studies at physiological pH. However, 5-OMe-DOM underwent a decrease during the acidification.
Usually, the molar absorption coefficient of the *trans* azonium is larger than that of the neutral *trans* form which is predicted by large oscillator strengths from TDDFT calculations.\textsuperscript{10} However, the relative size of the azonium band in this spectrum was significantly smaller since some of the compound was undergoing another process aside from simple protonation. The lack of a clear isosbestic point in the UV-Vis spectra in Figure 29b supports the observation that there is a more complex process occurring during the pH titration.

\textbf{Figure 29.} (a) Protonation of the *trans* form of 5-OMe-DOM (17) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the *trans* form of 17 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maximum (575 nm). The apparent pK\textsubscript{a} of the azonium ion was determined to be 7.9, as marked by the solid arrow. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene and the azonium, as calculated by HypSpec.

To observe the decrease of 5-OMe-DOM, the UV-Vis spectrum of the compound was monitored at a pH of 7.5 over several hours (Figure 30). Initially the experiment was run in 5 mM universal buffer and the absorption band of the azonium was observed to decrease over the course of several hours. The initial spectrum is indicated by the solid black line and the apparent decrease is shown by the spectra in grey, taken at 5 min intervals, until reaching a plateau (black dotted line, Figure 30a). The apparent decrease at the absorbance maximum of the azonium (577 nm) as a function of time is shown in Figure 30b. The universal buffer which the experiment was run in
is made of four components: CAPSO, TRIS, MES and NaOAc. To rule out the buffer components playing a role in the observed decrease, the experiment was re-run in sodium phosphate buffer (Figure 30c,d). The apparent decrease was reproducible in the sodium phosphate buffer which suggested that the buffer components were not playing a role in the observed spectral change. The process occurred over several hours and it took approximately 5 h for the absorbance maximum of the azonium to reach a plateau. Interestingly, the process reaches a point where it plateaus and therefore it cannot be an irreversible reaction or the decrease would continue until the compound had completely degraded.

Figure 30. Effect of buffer on the absorption spectra of the apparent decrease of 5-OMe-DOM (17) at a pH of 7.5 in 5 mM universal buffer (a) and 5 mM sodium phosphate buffer (c). Absorbance at the $\lambda_{\text{max}}$ as a function of time in 5 mM universal buffer (b) and 5 mM sodium phosphate buffer (d).

We then tested to see whether the solubility of the 5-OMe-DOM (17) in aqueous buffer was causing the observed decrease. Initially the experiments in the 5 mM universal buffer and 5 mM sodium phosphate buffer were run in with 5% DMSO to help solubilize the compound in the aqueous solutions. We first increased the percentage of DMSO to 30% in the 5 mM universal buffer solution and monitored the apparent decrease over several hours (Figure 31a). The absorbance as a function of the time is shown in Figure 31b. The absorbance maximum of the
azonium shifted from 577 nm with 5% DMSO to 624 nm with 30% DMSO; however, the decrease in the azonium was still observed. An experiment using 30% methanol to solubilize 5-OMe-DOM in the 5 mM universal buffer was then run. The UV-Vis spectra of the apparent decrease with 30% methanol are depicted in Figure 31c. The absorbance maxima of the azonium ion shifted to 603 nm in this solvent mixture. The absorbance as a function of time is depicted and a similar decrease in the absorbance of the azonium ion was observed (Figure 31d). Therefore, differing the solution composition did not appear to affect the absorbance decrease observed for 5-OMe-DOM. There are clear isosbestic points during these experiments which suggest that a new product with lower wavelength absorbance is forming.

![Graphs showing absorbance changes](image)

**Figure 31.** Effect of solubility on the absorption spectra of the apparent decrease of 5-OMe-DOM (17) in 5 mM universal buffer with (a) 30% DMSO at a pH of 7.5 and (c) 30% MeOH at a pH of 7.7. Absorbance at the $\lambda_{\text{max}}$ as a function of time in 5 mM universal buffer with (b) 30% DMSO at a pH of 7.5 and (d) 30% MeOH at a pH of 7.7.

Since the solubility of 5-OMe-DOM in the aqueous solution did not appear to affect the observed decrease, the dependence of the pH of the solution on the decrease was tested. When the experiment was run at a pH of 6, the amount of decrease in the azonium peak observed was significantly smaller (Figure 32a). Since the apparent $pK_a$ of 5-OMe-DOM was approximately
7.9, the compound was almost completely in the protonated azonium form. Therefore, it did not appear to be an acid-related degradation of the diazene or the observed decrease would have been expected to be larger as the solution was acidified. The experiment was then run at a slightly more basic pH of 8 to observe how the decrease would be affected (Figure 32c). The decrease of the azonium was even larger than was observed at a pH of 7.5. Since the decrease occurs in the azonium, the pH needs to be low enough for some azonium to be present. However, further acidification of the pH appears to minimize the absorbance decrease and therefore there is a pH window where the largest decrease occurs. Further experiments at an even lower pH to see if the process completely stops and a higher pH where only the neutral trans form of 5-OMe-DOM is present would help better understand the cause of the observed absorbance decrease.

**Figure 32.** Effect of pH on the absorption spectra of the apparent decrease of 5-OMe-DOM (17) in (a) pH=5.9 with 7% DMSO in 5 mM universal buffer and (c) pH=8.0 with 12% DMSO in water. Absorbance at the $\lambda_{\text{max}}$ as a function of time (b) pH=5.9 with 7% DMSO in 5 mM universal buffer and (d) pH=8.0 with 12% DMSO in water.
To compare the dependence of the pH on the apparent decrease of 5-OMe-DOM, the plots of the absorbance as a function of time were fit with a monoexponential decay curve. A summary of the half-lives ($\tau_{1/2}$) of the decay are tabulated in Table 2. Additionally, the percentage decrease in the absorbance of the azonium at the $\lambda_{\text{max}}$ over the course of the decay was calculated for each set of conditions. It was seen that the percentage of DMSO used to solubilize the compound did not greatly affect the observed $\tau_{1/2}$ of the decay. The half-life of 5-OMe-DOM decrease was determined to be 63 min with 5% DMSO and 67 min with 30% DMSO. Additionally, the percentage of DMSO did not affect the observed decrease of the maximal absorbance of the azonium. The solution of 5-OMe-DOM with 5% DMSO decreased by 45% and the solution with 30% DMSO decreased by 44% compared to the maximum absorbance of the azonium before the apparent decrease. Thus, slight differences in the concentration of DMSO should not affect the ability to compare the half-lives of the decay while varying the pH of the solutions. When the pH of the solution with 5-OMe-DOM was lowered to 5.9, the time the compound took to decay was slower (85 min) and the amount of observed decay decreased to 15% (Table 2). Therefore, it did not appear to be an increase in the acidity of the solution which was causing the observed process. When the solution was under more basic conditions at a pH of 8.0, the half-life of the process was longer (125 min) but the maximal absorbance of the azonium decreased by 64%, more than at a pH of 7.5. This sample was run in water; whereas the other experiments were in 5 mM universal buffer so the solution composition could have affected the observed decrease. The absorbance decrease occurs in the azonium band therefore the solution appears to need to be adequately acidic to protonate some of the compound, but further increasing the acidity minimizes the observed change.

**Table 2.** The monoexponential decay half-lives of the azonium of 5-OMe-DOM (17) in different pH solutions are tabulated.

<table>
<thead>
<tr>
<th>pH</th>
<th>DMSO (%)</th>
<th>Solution</th>
<th>Decay $\tau_{1/2}$</th>
<th>Decrease at $\lambda_{\text{max}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>5</td>
<td>5 mM universal buffer</td>
<td>63 min</td>
<td>45</td>
</tr>
<tr>
<td>7.5</td>
<td>30</td>
<td>5 mM universal buffer</td>
<td>67 min</td>
<td>44</td>
</tr>
<tr>
<td>5.9</td>
<td>7</td>
<td>5 mM universal buffer</td>
<td>85 min</td>
<td>15</td>
</tr>
<tr>
<td>8.0</td>
<td>12</td>
<td>water</td>
<td>125 min</td>
<td>64</td>
</tr>
</tbody>
</table>

Based on the UV-Vis spectra of 5-OMe-DOM after the apparent decrease in the azonium ion, it appeared that there was still an absorbance at the $\lambda_{\text{max}}$ of the azonium remaining which suggested that some of the compound was still intact. Thin-layer chromatography (TLC) was used to test
whether 5-OMe-DOM was present after being exposed to aqueous conditions where the absorbance decrease occurs (Figure 33). The pure sample (S) was compared to the “decreased” product (D). The retention factor (Rf) of both compounds in 10% MeOH/DCM was 0.7 and the blue colour of the samples on the TLC plate was consistent with some 5-OMe-DOM still being present after the observed absorbance decrease. The KMnO₄ stain did not show the 5-OMe-DOM, but it revealed that another product with a slightly lower Rf (0.64) was present after the absorbance decrease in water. Interestingly, in the aqueous solution, there were insoluble blue aggregates observed after the absorbance decrease. In the azonium form, TDDFT calculations predict that the azobenzene rings are planar and therefore the compounds could potentially be π-stacking with one another and forming insoluble aggregates. If this was the sole cause of the absorbance decrease, it would have been expected to increase as the pH was lowered since the azonium is in higher concentration. However, the opposite was observed. Therefore, there appears to be both an issue with solubility of 5-OMe-DOM in the aqueous solution and a process occurring which forms a new compound which is observed on the TLC plate only by KMnO₄ stain. The new product observed is hypothesized to be a hydrated species where hydroxide attacks the diazene. Since this would break the extended conjugation of the azobenzene, this could explain why this product was not observed under ambient light (Figure 33). The absorbance decrease was observed to be far greater under more basic conditions where there would be less azonium present than under acidic conditions where 5-OMe-DOM would be almost completely protonated. The pH of the solution could both affect the ability of the azonium ions to aggregate and the amount of hydration of the diazene. There is more hydroxide present in the more basic solution which could explain the greater observed decrease of the absorbance of the azonium. Further characterization of the absorbance decrease is necessary to better understand whether aggregation, hydration or another process are contributing to the loss of the azonium absorbance. Better understanding this apparent decrease will help to determine how to prevent it to allow in vivo experiments under physiological conditions using the DOM substitution pattern.
Figure 33. Thin-layer chromatography of 5-OMe-DOM (17) in 10% MeOH/DCM. The standard is “S” and the decrease product “D.” Visualized under (a) ambient light, (b) 254 nm UV light, and (c) KMnO₄ stain.

For 4-OMe-DOM (18), the bis-para-azetidine substituted derivative, the UV-Vis spectral data is presented in Figure 34. At a basic pH, the neutral trans form was found to have absorbance maxima at 481 nm and 432 nm (Figure 34b). Acidification of the compound generated the azonium ion which red-shifted the absorbance band to a maximum of 585 nm, with the tail extending out to 780 nm which suggests that the lone pair on the nitrogen of the azetidine ring was optimal for donation of electron density into the ring system. However, the compound underwent a large decrease in the absorbance during the pH titration. Based on the observation that the decrease of 5-OMe-DOM may be due to issues with aggregation of the compound in aqueous conditions, this compound may also form insoluble aggregates which would explain the observed decrease. Further experiments are needed to characterize whether 4-OMe-DOM undergoes the same apparent decrease as 5-OMe-DOM. The spectral properties of 4-OMe-DOM are red-shifted and therefore if the decrease could be prevented then it could be an interesting photoswitch for in vivo studies.
Figure 34. (a) Protonation of the trans form of 4-OMe-DOM (18) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the trans form of 18 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). The black line represents the absorption spectrum at physiological pH. The pKₐ of the protonation event was not determined as the azonium species underwent a significant decrease during the titration. The maximum wavelength of absorbance of the azonium ion occurred at 586 nm.

Comparing the spectral properties of the DOM-substituted azobenzenes coupled to 6-, 5-, and 4-membered bis-para-amino rings, it was seen that a decrease in ring size lead to an increase in the ability of the nitrogen lone pair to donate electron density into the aromatic ring system. This was expected since the steric hindrance of the para-substituent should decrease with ring giving a smaller dihedral angle and therefore increase resonance with the conjugated aromatic system. The spectral properties for comparison are tabulated in Table 3. Decreasing the bis-para-amino ring size from 6-membered to 5-membered increased the observed absorbance maxima in both the neutral trans form as well as in the trans azonium. The absorbance band of the azonium also extended significantly further into the near-IR (775 nm compared to 720 nm). The higher pKₐ of 7.9 for 5-OMe-DOM compared to 5.2 for 6-OMe-DOM also suggests that the para-pyrrolidino substituents better participate in the extended conjugation. The increase in electron density of the ring aids the azonium formation at a higher pH. However, despite the optimal spectral properties of 5-OMe-DOM, the apparent decrease observed under physiological conditions needs to be resolved before it could be useful for in vivo studies. The 4-OMe-DOM derivative had similar
properties to 5-OMe-DOM but it underwent an even more prominent decrease during the pH titration and only a small amount of the red-shifted azonium ion formed. Since the spectral properties are similar, the 5-membered and 4-membered rings likely participate in the extended conjugation to similar extents. The two derivatives have small predicted dihedral angles of 6° for 5-OMe-DOM and 2° for 4-OMe-DOM (Table 3). The 6-OMe-DOM derivative, however, has a predicted dihedral angle of 17° and therefore the increased steric hindrance with the ring must be preventing it from resting close as close to planar and therefore the observed spectral properties are less red-shifted. While the long wavelength absorbance of the azonium of 5-OMe-DOM was promising, along with the near-neutral apparent pK_a, the decrease which was observed during the pH titration was concerning. Therefore, we wanted to extend the library to include another derivative which would sterically be similar to the 5-membered ring but which would be more conducive to testing under physiological conditions. TDDFT calculations predicted that the dihedral angle of a diethyl substituent would be 11°, in between that of the pyrrolidino (6°) and piperidino (17°) derivatives and therefore should have a more red-shifted absorbance than 6-OMe-DOM but circumvent the stability or solubility issue of 5-OMe-DOM (Table 3).

**Table 3.** TDDFT predicted (B3LYP (6-31++G**)) and experimentally observed spectral properties for DOM-substituted azobenzene derivatives.

<table>
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<tr>
<th>Compound</th>
<th>Predicted λ_{max} (trans neutral form) (nm)</th>
<th>Observed λ_{max} (trans neutral form) (nm)</th>
<th>Predicted λ_{max} (trans azonium form) (nm)</th>
<th>Observed λ_{max} (trans azonium form) (nm)</th>
<th>Maximum λ absorbance tail (nm)</th>
<th>Predicted dihedral angle (°)</th>
<th>Apparent pK_a of azonium</th>
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The compound diEt-DOM (19) was synthesized using an analogous Buchwald-Hartwig cross coupling of diethylamine with the diBr-DOM (15) starting material. The spectral properties of this derivative are reported in Figure 35. The neutral trans form which was present under basic conditions (blue) in Figure 35b had absorbance maxima at 470 nm and 383 nm. Acidification produced the azonium ion which had a λ_{max} of absorption at 542 nm with the band extending out to 720 nm. Further acidification lead to double protonation (azonium plus ammonium) of the parent azobenzene which hypsochromically shifted the λ_{max} of absorption to 490 nm. The apparent pK_a of the azonium ion was determined to be 7.1 and the apparent pK_a of the doubly protonated species was found to be 6.2. Since the pK_a’s of the two protonation events are similar,
the absorbance maximum of the azonium ion was difficult to determine from the UV-Vis spectra of the pH titration. The deconvoluted spectra for the azonium (AH⁺, Figure 35d) was therefore used to determine the λ_max of the absorbance band. Comparing this derivative to 5-OMe-DOM, the absorbance maximum of the azonium ion was at a much shorter wavelength of 542 nm for diEt-DOM compared to 575 nm for 5-OMe-DOM despite the TDDFT prediction of 595 nm for both compounds (Table 3). The wavelength to which the tail of the absorbance band extended was 720 nm for diEt-DOM compared to 775 nm for 5-OMe-DOM. Therefore, despite the prediction that these compounds would have similar spectral properties, the experimental results showed that the bis-para-diethyl group donated less electron density into the aromatic ring system and had less promising red-shifting than the bis-para-pyrrolidine counterpart. The observed spectral properties had closer similarity to 6-OMe-DOM.

![Figure 35](image)

**Figure 35.** (a) Protonation of the *trans* form of diEt-DOM (19) generates the azonium ion. (b) The pH dependence of the UV-Vis spectra of the *trans* form of 19 in aqueous buffer. The spectrum at the highest pH is shown in blue. The azonium ion is produced as the pH is lowered, as shown by the series of grey solid lines until a maximum amount of azonium is obtained (red line). Further decreasing the pH produced the doubly protonated species (azonium plus ammonium), indicated by the dotted grey lines growing to a maximum concentration (dotted red line). The black line represents the absorption spectrum at physiological pH. (c) The pH dependence of the azonium ion versus the absorbance at the absorbance maximum (560 nm). The pK_a of the azonium formation was determined to be 7.1 and the pK_a for the doubly protonated species was 6.2. The solid arrow marks the pK_a of the azonium ion. (d) The deconvoluted UV-Vis spectra of the neutral azobenzene, the azonium and the doubly protonated species, as calculated by HypSpec.
Comparing the spectral properties of the TOM-substituted (Table 1) versus the DOM-substituted (Table 3) azobenzenes, there were some general trends observed. While the absorbance maxima of the neutral trans forms appeared to be similar for the two substitution patterns, the trans azonium had red-shifted absorbance maxima by 15 nm to 40 nm for the DOM compared to the TOM derivatives. This observation was consistent with TDDFT predictions that the absorption spectrum of the trans azonium would have a small bathochromic shift (Table 1, Table 3). The absorbance bands of the trans azonium form of the DOM-substituted derivatives also had tails which extended 10 nm to 70 nm further into the near-IR. Despite the further red-shifting of the absorption spectra for the DOM derivatives, however, the apparent pKₐ’s were lower than for the TOM counterparts. A higher concentration of acid was necessary for them to undergo protonation which suggests that the azonium is less stable in the DOM derivatives. This is likely due to an increase in the dihedral angle between the para substituents and the azobenzene ring system since it has a meta substitution. The predicted dihedral angles followed this trend where the predicted angles were smaller for the TOM derivatives: 6° for 6-OMe-TOM and 4° for 5-OMe-TOM. The predicted dihedral angles were larger for the DOM derivatives: 17° for 6-OMeDOM and 6° for 5-O Ме-DOM. Thus, since the lone pair of electrons on the nitrogen atom of the para substituents cannot donate as much electron density into the aromatic system, the pKₐ is lowered. However, the placement of the auxochromes gives the DOM derivatives a further red-shift as was expected based on the studies by Wizinger and colleagues. The lower pKₐ of the trans azonium of the DOM derivatives is ideal for their use in vivo. Woolley and colleagues showed that trans-to-cis isomerization of TOM-substituted azonium ions is often accompanied by deprotonation since the geometry of the cis isomer prevents formation of the intramolecular H-bond with the proximal ortho-methoxy substituents and therefore the cis azonium has a lower pKₐ. They reported that the cis azonium for the TOM-substituted azobenzene had a pKₐ approximately 1.5 units lower than the trans azonium. This deprotonation accompanying the isomerization drastically increases the relaxation time since the neutral cis form has more double bond character of the diazene bond and therefore the isomerization is slower. Thus, there is a complex equilibrium of four states: neutral trans, neutral cis, trans azonium and cis azonium (Figure 36). This slower thermal relaxation is ideal for in vivo biological testing since it is necessary to accumulate a large fraction of the cis isomer for a large
biological response to be observed. Thus, the pK\textsubscript{a} of the trans azonium would ideally be near-neutral such that only the trans form is protonated.

![Diagram of isomerization and protonation states of a model compound.](image)

**Figure 36.** Isomerization and protonation states of a model compound.

Comparing the dioxane-ortho-methoxy to the ortho-meta-methoxy substitution pattern previously studied by Woolley and colleagues,\textsuperscript{10} the absorbance maxima of the trans azonium forms were less red-shifted. For the DOM substitution pattern coupled to bis-para-piperidine rings, the \( \lambda_{\text{max}} \) of the trans azonium was 560 nm. While this was red-shifted compared to the TOM-substituted derivatives, the ortho-meta-methoxy substituted compound with bis-para-piperazine rings had an absorbance maximum bathochromically shifted to 630 nm.\textsuperscript{10} However, the DOM-substituted derivative has a much high pK\textsubscript{a} of 5.2 compared to 2.6 for the ortho-meta-methoxy substitution. Additionally, retaining the tetra-ortho substitution of the diazene in the DOM substitution pattern should slow the rate of thermal relaxation compared to the ortho-meta-methoxy substituted compounds. Therefore, while the absorbance is less red-shifted, the higher pK\textsubscript{a}’s and the likely slower thermal relaxation times make DOM an interesting substitution pattern for further studies.
3.3 Conclusions

The novel dioxane-ortho-methoxy (DOM) substitution pattern was incorporated into a series of compounds with various bis-para-amino substituents and compared to the tetra-ortho-methoxy substituted versions. The DOM-substituted derivatives had red-shifted absorbance maxima of the trans azonium ions and the tail of the azonium band extended further into the near-IR compared to the TOM-substituted counterparts. The red-shifted properties make DOM an interesting photoswitch since the optimal tissue penetration occurs in the near-IR and therefore the compounds need to absorb long-wavelength light for photo-control in vivo. While the DOM-substituted derivatives were not as red-shifted as the ortho-meta-methoxy substituted compounds previously studied by Woolley and colleagues, it was predicted that the DOM-substituted compounds would have slower thermal relaxation times since the tetra-ortho substitution was retained which was found to be necessary for longer relaxation times.\textsuperscript{10}

Initially the starting material diBr-DOM was coupled to 6-, 5- and 4-membered bis-para-amino rings. The ability of the para-substituents to donate electron density into the conjugated aromatic system was predicted by the dihedral angle. A small dihedral angle allowed the lone pair on the nitrogen atom to better donate electron density into the ring system and therefore the UV-Vis spectra underwent a bathochromic shift. The TDDFT predicted dihedral angles for 6-OMe-DOM, 5-OMe-DOM and 4-OMe-DOM were 17°, 6° and 2° respectively. The smaller dihedral angles in the 5- and 4-membered ring derivatives allowed the nitrogen to better participate in conjugation with the ring system and therefore the absorbance maxima and the tail of the absorbance band were further red-shifted than 6-OMe-DOM. This was anticipated since the larger para ring in 6-OMe-DOM would have more steric hindrance with the proximal aromatic ring and the meta-oxygen which would prevent it from lying as close to planar. Although 5-OMe-DOM and 4-OMe-DOM had ideal UV-Vis spectral properties, both of these derivatives underwent a progressive decrease in the absorbance at the $\lambda_{\text{max}}$ of the azonium ion under aqueous conditions during the pH titration.

The absorbance decrease of 5-OMe-DOM was measured under various conditions to characterize the source of the decrease. It was not an irreversible process since the absorbance of the azonium decreased and then plateaued with some of the absorbance remaining. The
components of the buffer were not found to play a role in the observed process and the solvent (DMSO or MeOH) used to solubilize the 5-OMe-DOM did not prevent the observed decrease. The pH dependence of the decrease was analyzed and a larger decrease in the absorbance of the azonium was observed at a higher pH, despite their being less azonium present. Since the UV-Vis spectra indicated that some azonium remained even after the absorbance decrease, TLC was used to confirm that the compound was partially intact after the observed decrease. The retention factor and the colour of the spot on TLC confirmed that the compound was still present; however, insoluble blue aggregates were seen in the solution which suggested that the solubility of the compound may be influencing the observed process. Woolley and colleagues previously reported that the azonium ion of the tetra-ortho-methoxy substituted azobenzene is planar. Therefore, this could allow 5-OMe-DOM to form π-stacking interactions and precipitate out of the solution causing the observed decrease. Additionally, a spot of lower Rf was observed by KMnO₄ stain which could indicate that another product is forming during the decrease. It is hypothesized that this could be a hydration product of the diazene which breaks the conjugation and therefore the compound is not visualized under ambient or UV light. This would be consistent with the observation that the decrease is far greater under more basic pHs despite the concentration of azonium being lower. Further studies need to be performed to determine whether aggregation, hydration or another process is causing the observed decrease and to determine conditions under which this process will not happen. If aggregation is as a result of too high of a concentration of the azonium species being present in the solution, UV-Vis spectra could be measured in a 10 cm cuvette. This would permit a far more dilute sample to be used which potentially would avoid aggregation of the compound. The observed decrease of 4-OMe-DOM during the pH titration was very large; it would be interesting to characterize whether solubility or another process is causing this decrease and if the decrease behaves in a similar way to 5-OMe-DOM. Since 4-OMe-DOM had the furthest red-shifted spectral properties, the source of the decrease should be determined to permit further testing of this derivative.

Since 5-OMe-DOM underwent an absorbance decrease under physiological conditions, we looked for another para-substituent to use in place of the 5-membered pyrrolidine rings which would have a similar dihedral angle but avoid the observed decrease. TDDFT calculations predicted that the dihedral angle of bis-para-diethyl (diEt-DOM) would be 11° compared to 6°
for 5-OMe-DOM. Therefore, the nitrogen of the diethyl substituent was predicted to be slightly less electron donating. The observed spectral properties of diEt-DOM were found to be far less red-shifted than the calculations predicted so we are interested in keeping a 5-membered or 4-membered N-containing ring in the \textit{para}-position to benefit from the bathochromic shift of the \textit{trans} azonium. We are currently expanding the library of 5-membered DOM-substituted derivatives to alter the solubility while retaining the red-shifted spectral properties (Figure 37).

\begin{center}
\includegraphics[width=0.5\textwidth]{derivative.png}
\end{center}

\textbf{Figure 37.} Future DOM-substituted derivatives to synthesize.

Once a stable and soluble DOM-substituted derivative with ideal red-shifted spectral properties is found, the ability of these compounds to undergo long-wavelength photoswitching needs to be characterized. Woolley and colleagues previously reported that tetra-\textit{ortho}-methoxy substituted derivatives with bis-\textit{para}-aminos undergo robust photoswitching with 635 nm light\textsuperscript{5} or even 660 nm light\textsuperscript{10}. Thus, the DOM-substituted derivatives, which have red-shifted absorbance maxima of the azonium compared to the TOM counterparts could undergo photoswitching with even longer wavelength light. Photo-control of these photoswitches with long wavelength light is necessary for them to be useful for \textit{in vivo} modulation of biomolecules since the optimal wavelengths of light to penetrate into tissues occurs in the near-IR.
3.4 References


Appendix

Spectral data for synthesized compounds:

- $^1$H NMR
- $^{13}$C NMR
- Mass spectra
$^1$H NMR 400 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (CDCl$_3$)
MS DART (ESI⁺)

Chemical Formula: C₈H₁₀NO₄⁺
Exact Mass: 184.06
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$^{13}$C NMR 125 MHz (CDCl$_3$)
MS DART (ESI⁺)

Chemical Formula: C₈H₁₂NO₂⁺
Exact Mass: 154.09
$^1$H NMR 400 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (CDCl$_3$)
MS DART (ESI⁺)

Chemical Formula: C₈H₁₁BrNO₂⁺
Exact Mass: 232.00
$^{1}H$ NMR 500 MHz (CDCl$_3$)
MS Q-TOF (ESI⁺)

Chemical Formula: C₁₅H₁₇Br₂N₂O₄⁺
Exact Mass: 458.95
$^{13}$C NMR 125 MHz (Methanol-$d_4$)
MS Q-TOF (ESI+)

Chemical Formula: C_8H_{18}NO_2^+
Exact Mass: 160.13
$^1$H NMR 500 MHz (Methanol-$d_4$)
MS Q-TOF (ESI+)
$^1$H NMR 500 MHz (Methanol-$d_4$)
MS Q-TOF (ESI+)}

Chemical Formula: $C_{32}H_{52}N_6O_6^{2+}$
Exact Mass: 616.3937
m/z: 308.1969 (100.0%), 308.6986 (34.6%), 309.2002 (5.8%), 308.6954 (2.2%), 309.1990 (1.2%)
MS Q-TOF (ESI+)

Chemical Formula: C\textsubscript{32}H\textsubscript{52}N\textsubscript{6}O\textsubscript{6}\textsuperscript{2+}
Exact Mass: 616.3937
m/z: 308.1969 (100.0%), 308.6986 (34.6%), 309.2002 (5.8%), 308.6954 (2.2%), 309.1990 (1.2%)
$^{1}H$ NMR 500 MHz (Acetone-$d_{6}$)
$^{13}\text{C NMR}$ 125 MHz (Acetone-$d_6$)
MS Q-TOF (ESI+)
$^1$H NMR 500 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (D$_2$O)
**MS Q-TOF (ESI⁺)**

Chemical Formula: C_{40}H_{66}N_{8}O_{6}^{2+}

Exact Mass: 754.5094

m/z: 377.2547 (100.0%), 377.7564 (43.3%), 378.2581 (9.1%), 377.7533 (3.0%), 378.2549 (1.3%), 378.7598 (1.3%), 378.2569 (1.2%)
**MS Q-TOF (ESI+)**

Chemical Formula: \( \text{C}_{40}\text{H}_{66}\text{N}_{8}\text{O}_{6}^{2+} \)

Exact Mass: 754.5094

\( m/z \) values:
- 377.2547 (100.0%), 377.7564 (43.3%), 378.2581 (9.1%), 377.7533 (3.0%), 378.2549 (1.3%), 378.7598 (1.3%), 378.2569 (1.2%)

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Exact Mass: 529.3021
MS Q-TOF (ESI⁺)

Chemical Formula: $C_{28}H_{41}N_{4}O_{6}^+$
Exact Mass: 529.3021
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![NMR spectrum of compound 13](image)
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Chemical Formula: C_{26}H_{37}N_{4}O_{6}^{+}
Exact Mass: 501.2708
MS Q-TOF (ESI+)

Chemical Formula: $C_{26}H_{37}N_4O_6^+$

Exact Mass: 501.2708
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MS Q-TOF (ESI+)

Chemical Formula: C_{24}H_{33}N_{4}O_{6}^+

Exact Mass: 473.2395
MS Q-TOF (ESI+)

Chemical Formula: C_{24}H_{33}N_{4}O_{6}^+
Exact Mass: 473.2395
$^1$H NMR 500 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (CDCl$_3$)
MS Q-TOF (ESI⁺)

Chemical Formula: C₃₀H₄₁N₄O₈⁺
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Exact Mass: 585.2919

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<th>$\Delta m$/ (Da)</th>
<th>$\Delta m$/ (ppm)</th>
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<th>MFG Score</th>
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**Predicted Isotope Match Table**

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<th>Abund (%)</th>
<th>Calc Abund (%)</th>
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$^1$H NMR 500 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (CDCl$_3$)
MS Q-TOF (ESI+)

Chemical Formula: C_{28}H_{36}N_{4}O_{8}^+

Exact Mass: 557.2606
MS Q-TOF (ESI⁺)

Chemical Formula: C₂₈H₃₇N₄O₈⁺
Exact Mass: 557.2606

Target Ion Species

<table>
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<tr>
<th>Ion Species</th>
<th>m/z</th>
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MFG Calculator Results

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<th>Target m/z</th>
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<th>t⁺/⁻ (mDa)</th>
<th>t⁺/⁻ (ppm)</th>
<th>DBE</th>
<th>MFG Score</th>
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Predicted Isotope Match Table

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<th>Abund (%)</th>
<th>Calc Abund (%)</th>
<th>t⁺/⁻</th>
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$^{1}H$ NMR 500 MHz (DMSO-$d_6$)
$^{13}$C NMR 125 MHz (DMSO-$d_6$)
**MS Q-TOF (ESI⁺)**

Chemical Formula: C\(_{28}\)H\(_{39}\)N\(_4\)O\(_8\)⁺

Exact Mass: 529.2293
**MS Q-TOF (ESI*)**

Chemical Formula: $C_{26}H_{33}N_4O_8^+$
Exact Mass: 529.2293
$^1$H NMR 500 MHz (DMSO-d$_6$)
$^{13}$C NMR 125 MHz (DMSO-$d_6$)
MS Q-TOF (ESI+)

Chemical Formula: C_{28}H_{37}N_{4}O_{6}^+  
Exact Mass: 501.2708
MS Q-TOF (ESI$^+$)

**Chemical Formula:** $C_{28}H_{37}N_4O_6^+$  
**Exact Mass:** 501.2708
$^1$H NMR 500 MHz (CDCl$_3$)
$^{13}$C NMR 125 MHz (CDCl$_3$)
**MS Q-TOF (ESI⁺)**

Chemical Formula: $\text{C}_{26}\text{H}_{33}\text{N}_{4}\text{O}_{8}^+$

Exact Mass: 529.2293
**MS Q-TOF (ESI⁺)**

**Target Ion Species**

<table>
<thead>
<tr>
<th>Ion Species</th>
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<th>Ionic Formula</th>
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**MFG Calculator Results**

<table>
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<th>Target m/z</th>
<th>Ionic Formula</th>
<th>Calc m/z</th>
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<th>MFG Score</th>
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**Chemical Formula**: C_{26}H_{33}N_{4}O_{8}⁺

**Exact Mass**: 529.2293

**Predicted Isotope Match Table**

<table>
<thead>
<tr>
<th>Isotope m/z</th>
<th>Calc m/z</th>
<th>Diff (mDa)</th>
<th>Abund (%)</th>
<th>Calc Abund (%)</th>
<th>±/−</th>
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