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# Red-Shifting Azobenzene Photoswitches for in Vivo Use

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**CONSPECTUS:** Recently, there has been a great deal of interest in using the photoisomerization of azobenzene compounds to control specific biological targets in vivo. These azo compounds can be used as research tools or, in principle, could act as optically controlled drugs. Such "photopharmaceuticals" offer the prospect of targeted drug action and an unprecedented degree of temporal control.

A key feature of azo compounds designed to photoswitch in vivo is the wavelength of light required to cause the photoisomerization. To pass through tissue such as the human hand, wavelengths in the red, far-red, or ideally near infrared region are required. This Account describes our attempts to produce such azo compounds.

Introducing electron-donating or push/pull substituents at the *para* positions delocalizes the azobenzene chromophore and leads to long wavelength absorption but usually also lowers the thermal barrier to interconversion of the isomers. Fast thermal relaxation means it is difficult to produce a large steady state fraction of the *cis* isomer. Thus, specifically activating or inhibiting a biological process with the *cis* isomer would require an impractically bright light source.

We have found that introducing substituents at all four *ortho* positions leads to azo compounds with a number of unusual properties that are useful for in vivo photoswitching. When the *para* substituents are amide groups, these tetra-*ortho* substituted azo compounds show unusually slow thermal relaxation rates and enhanced separation of n−$\pi^*$ transitions of *cis* and *trans* isomers compared to analogues without ortho substituents. When *para* positions are substituted with amino groups, *ortho* methoxy groups greatly stabilize the azonium form of the compounds, in which the azo group is protonated. Azonium ions absorb strongly in the red region of the spectrum and can reach into the near-IR. These azonium ions can exhibit robust *cis*−*trans* isomerization in aqueous solutions at neutral pH.

By varying the nature of *ortho* substituents, together with the number and nature of *meta* and *para* substituents, long wavelength switching, stability to photobleaching, stability to hydrolysis, and stability to reduction by thiols can all be crafted into a photoswitch. Some of these newly developed photoswitches can be used in whole blood and show promise for effective use in vivo. It is hoped they can be combined with appropriate bioactive targets to realize the potential of photopharmacology.

## *cis*−*trans* PHOTOISOMERIZATION OF AZOBENZENE APPLIED TO BIOLOGY

In 1937, Hartley discovered that shining UV light on *trans* azobenzene produced the *cis* isomer.[1] Leaving the molecule in the dark, or irradiating it with blue light, recovered the thermodynamically more stable *trans* isomer. This process occurs efficiently, without major side reactions, and continues to occur when azobenzene is derivatized in numerous ways.[2] The robustness of the photoisomerization process, together with the relative ease of synthesis of many derivatives, has made azobenzene a popular photoswitch with applications in diverse fields from materials science to neurobiology.[3]

In a remarkable series of papers in the late 1960s, Erlanger and co-workers applied azobenzene to the photocontrol of enzymes and ion channels.[4,5] He did this by modifying ligands or inhibitors or even allosteric activators[6] with azobenzene units. These papers are written from the point of view of developing model systems to understand photoregulated processes that occur in nature. The idea of using these compounds as tools to manipulate biology seems to have developed later. For example, the Cys-reactive tethered ligands that Erlanger et al. developed for the acetylcholine receptor[5] were used in ground-breaking studies by Lester and Gurney to explore the biophysics of ion channel function. Based on this work and other work with caged compounds, the potential for using light to manipulate localized, mostly ex vivo, physiological processes was reviewed by Lester and Gurney in the 1980s.[7]

With advances in molecular and structural biology, it has now become possible to tether azobenzene derivatives site-

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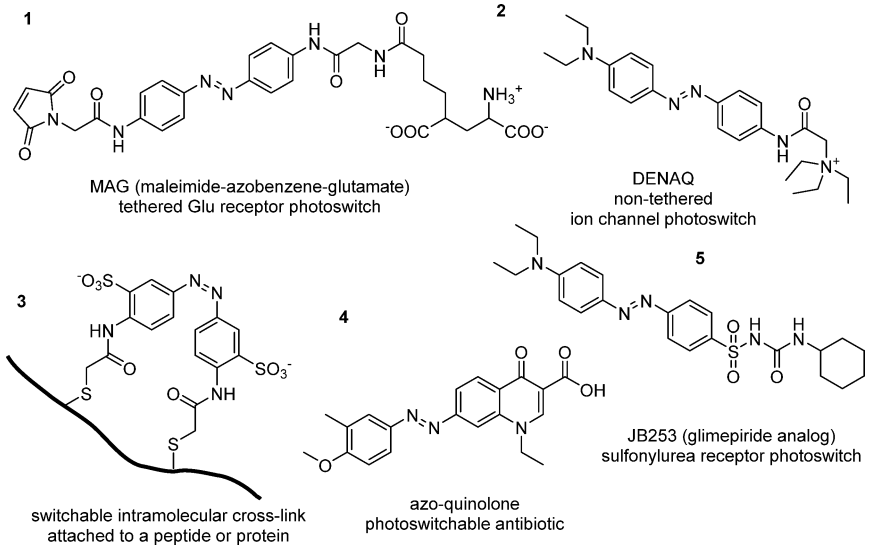


**Figure 1.** Examples of azobenzene derivatives that act on biomolecules. For references, see **1**,[13] **2**,[28] **3**,[29,30] **4**,[24] and **5**.[25]

specifically to essentially any protein target in living cells, and in some cases in vivo (e.g., in transparent zebrafish).[8] This can be accomplished via reaction with a Cys residue introduced via site-directed mutagenesis, or using biorthogonal ligation methods together with nonsense codon suppression strategies.[9−12] Targeted protein modification with azobenzenes has now led to light switchable Glu receptors,[13,14] $K^+$ channels,[9] acetylcholine receptors,[15] and GABA receptors[16] as well as kinases,[12] kinesins,[17] and transcription factors.[18] (Figure 1).

In parallel with these developments has been the realization that drugs could be made light sensitive via incorporation of an azobenzene unit.[19−21] "Azologized" drugs now include the GABA receptor potentiator propofol,[22] the opioid receptor agonist fentanyl,[23] antibacterial quinolones,[24] and ATP-sensitive potassium channel active sulfonylureas (Figure 1).[25] While it remains to be seen how such drugs will behave in whole organisms, the possibility of remote in vivo optical control of a drug is a tantalizing possibility. It directly addresses a primary problem of drug design by making the drug active only where and when light is applied. "Photopharmacology", a term that appeared in a science fiction context in the 1970s,[26] has now become a real possibility.[21,27]

## AZOBENZENES IN VIVO

For an azobenzene photoswitch to operate in vivo, a number of criteria have to be met. Besides being nontoxic, the switch must be stable to hydrolysis and to reduction of the azo group, which would render it inoperable. In vivo reduction of the azo group of one azo dye (4-[(2,4-diaminophenyl)azo] benzenesulfonamide) played a starring role in the early days of pharmaceutical chemistry. This dye, trademarked Prontosil, was discovered by Domagk in 1932 to cure bacterial infections.[31] The discovery of Prontosil earned Domagk the Nobel Prize in Physiology or Medicine in 1939. Scientists at the Pasteur Institute in Paris then found that sulfanilamide, produced by reductive cleavage of the azo group of Prontosil by bacterial enzymes in the gut, was the active antibacterial agent (not Prontosil itself). This discovery, in turn, led to the era of the "sulfa drugs".[31]

What happened with Prontosil perhaps led to the widespread notion that azobenzenes are reduced in vivo. However, not all azobenzenes are metabolized in the same manner as Prontosil. The azo compound 2,6-diamino-3-(phenylazo)pyridine (trade name Pyridium) is used as a local analgesic for symptomatic relief of urinary tract infections and is taken orally, and the azo bond is relatively stable (although hydroxylation on the aromatic rings occurs).[32] The metabolism of many azobenzenes has been analyzed by scientists interested in the effects of widely used azo-based food dyes. It varies greatly with the structure of the dye and the route of administration (e.g., whether it is exposed to gut bacteria).[33] This body of knowledge is likely to be very useful in guiding the development of photopharmaceuticals.

To our knowledge, photoswitching has not been considered in any of these studies, only the metabolism and toxicity of the stable (*trans*) forms of the dyes. To address in vivo stability of photoswitching, we monitored photoswitching of a bis-*p*-amido substituted azobenzene using a fluorescent reporter assay and found photoswitching behavior was retained for days in developing zebrafish.[34] Bis-*p*-amido substituted azobenzene is the core of several photoswitches that have been developed to target biomolecules (Figure 1). Newly developed switches are now tested for their stability to reduction by glutathione, the predominant intracellular reductant. Some switches are stable and some are not, depending on the specific structure of the switch in question.[35,36] The widely held concern that all azobenzenes would be reduced in vivo[37] is too simplistic a view.

Aside from stability in vivo, a key feature that will determine the success of azobenzene-based tools for biological research and photopharmacology is the wavelength required to cause photoisomerization. With the exception of the eye (where azobenzene-based drugs to treat certain types of blindness are making real progress[28]), the body is opaque throughout most of the visible range. The UV light needed to cause *trans*-to-*cis* isomerization of unmodified azo compounds is strongly absorbed and scattered by skin. In principle, one can introduce light via fiber optics, as is done in optogenetics, but this is cumbersome. A straightforward solution is to use light in a wavelength range where penetration though body tissue is orders of magnitude better, that is, in the red to near-infrared (near-IR) region.[38]

This Account is a narrative describing our attempts to make azobenzene photoswitches that operate in this optical window. A recent review by Bléger and Hecht provides a more


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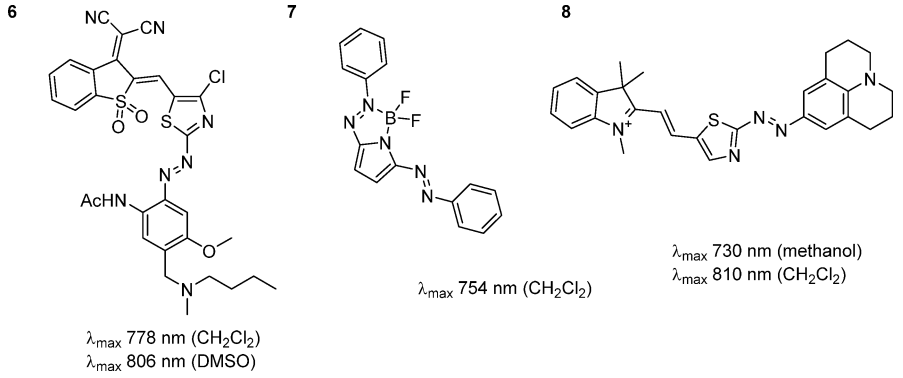


**Figure 2.** Some long wavelength azo dyes. For references, see **6**,[41] **7**,[42] and **8**.[43]

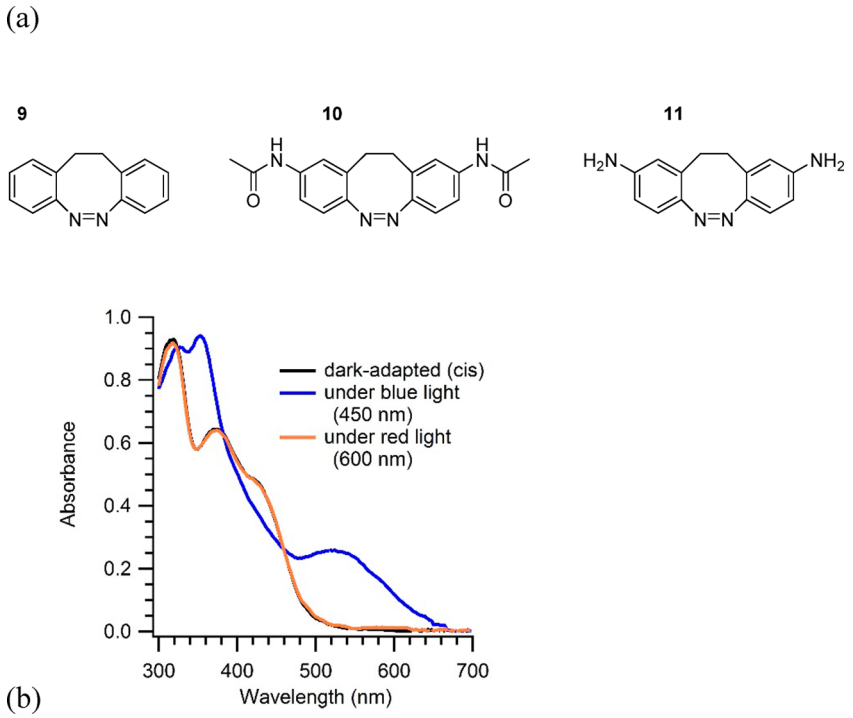


**Figure 3.** (a) Amino-substituted bridged azobenzene photoswitches. (b) UV−vis spectra of **11** obtained in aqueous solution under dark-adapted conditions and under blue (445 nm) and red (600 nm) irradiation.

comprehensive account of different classes of visible light activated photoswitches.[39]

## SOME GENERAL ISSUES FOR LONG WAVELENGTH PHOTOSWITCH DESIGN

In general, shifting azobenzene absorbance to longer wavelengths leads to faster thermal relaxation rates.[40] Sometimes fast thermal relaxation is useful. For instance, if one is triggering an ion channel involved in vision, rapid turn-off is critical for real-time responses.[28] In other cases though, one might wish to use a pulse of light to produce a long-lived *cis* isomer or simply to produce a large fraction of the *cis* isomer. Rapid thermal relaxation means that the steady state fraction of the *cis* isomer is small unless bright light sources with photon fluxes comparable to the thermal back reaction rate are used.

In addition to lowering the thermal barrier to isomerization, modifications to azobenzene designed to enable long wavelength switching can alter the relative stabilities of the ground state *cis* and *trans* isomers. An extreme case would be if *cis* and *trans* isomers became isoenergetic and were separated by a small thermal energy barrier. Such a photoswitch would be unable to drive conformational switching of a target biomolecule. Instead, conformational preferences of the target molecule would drive thermal equilibration of the switch. Thus, in designing red/near-IR photoswitches, it is important to consider the effect of modifications on the relative energies of the *cis* and *trans* isomers as well as the barrier between them.

## AZO DYES THAT ABSORB AT LONG WAVELENGTHS: PUSH−PULL SYSTEMS

Many thousands of azo dyes have been synthesized since the discovery of azobenzene in 1834. A number of dyes have been found that absorb in the red region and even the near-IR.[41−43] A few of these are shown in Figure 2. Although the structures of these dyes vary considerably, long wavelength absorption has typically been achieved by creating a push−pull system with electron donating groups on one side of the azo unit and electron withdrawing groups on the other. Because of their polarized structure, the absorption properties of these dyes are highly solvent dependent. Historically, most scientists inter-


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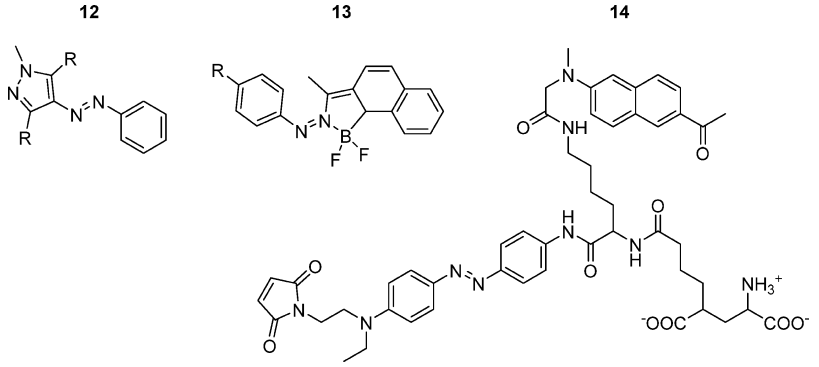


**Figure 4.** Examples of an azoheterocyclic switch (**12**), a $BF_2$ adduct (**13**), and a two-photon absorbing photoswitch (**14**).

ested in azo dyes did not study possible isomerization events. Photochromism would be an unwanted feature in a dye where color fastness is usually prized. It is likely that these long wavelength dyes have very short-lived *cis* isomeric forms. As a result, photoisomerization would not be observable without specialized laser flash photolysis apparatus. The Velasco group has studied the thermal relaxation behavior of push−pull azobenzene systems in detail[40] and has reported dyes that show thermal relaxation times as fast as 40 ns.[44]

The structures shown in Figure 2 are not easily adapted as components of photoswitches such as MAG (compound **1**, Figure 1) or as components of intramolecular cross-linkers (e.g., compound **3**, Figure 1) where the simple bis-*p*-amido azobenzene core has been used. Photoswitches usually require few rotatable bonds in order to preserve the end-to-end distance change that occurs upon isomerization.[45,46] In addition, the large size of these dyes may be a liability in pharmacological applications where the overall size of a drug is an important feature. Finally, the push−pull design of these long wavelength azobenzenes gives the molecule an inherent asymmetry. For photopharmacology, asymmetry is not a concern, but for an intramolecular cross-linker (compound **3**, Figure 1) having an asymmetric structure would also require two distinct bio-orthogonal means of attachment to a target protein to avoid the formation of distinct regioisomers.

## AZO DYES THAT ABSORB AT LONG WAVELENGTHS: C2 BRIDGED AZOBENZENES (DIAZOCINES)

In 2009, Siewersten et al.[47] reported the remarkable photochemical properties of a C2 bridged azobenzene (**9**). The *cis* and *trans* n−$\pi^*$ transitions of this compound are separated by some ∼100 nm making photochemical switching unusually complete in either direction. We made a bis-*p*-amido substituted derivative (**10**) to allow conjugation to biomolecules,[48] and in doing this we also made the bis-*p*-amino substituted version (**11**). The Herges group has reported similar molecules with amino groups in the 3,3′ positions.[49] This molecule (**11**) showed significant absorbance in the red region of the spectrum (Figure 3) in its *trans* isomeric form. Irradiation with red light (600 nm) produced full conversion to the *cis* isomer (Figure 3). The thermal relaxation rate was on the order of minutes at room temperature in aqueous solution. The drawback for in vivo use is that, because of the bridge, the *cis* state (the state produced by red light in this case) is the thermodynamically more stable form. Unless a photoswitch is truly bistable, that is, thermal relaxation is negligible on biological time scales, it is most convenient if the more stable state is the biologically inactive isomer. Otherwise, in the body, in the dark, there is a steady thermal production of the bioactive isomer. With compound **11**, blue light is needed to produce the less stable *trans* form. In addition to this limitation, the synthesis of these C2 bridged molecules proved quite challenging. However, substantial improvements in synthetic methods have now been reported.[50,51] Perhaps if their thermal relaxation rates could be slowed further, these compounds should be revisited for photopharmacological applications.

## AZO DYES THAT ABSORB AT LONG WAVELENGTHS: HETEROCYCLES, $BF_2$ ADDUCTS, AND TWO-PHOTON CHROMOPHORES

Although numerous heterocyclic azo compounds have been studied as dyes, these compounds are relatively underexplored as photoswitches. A recent study by the Fuchter group highlights the possibility that novel switching behavior can be obtained with such systems.[52] These authors studied arylazopyrazoles (**12**) and found thermal *cis* half-lives of days together with a large separation of the absorption maxima of the *cis* and *trans* isomers.

The Aprahamian group discovered that $BF_2$-adducts of azobenzenes (**13**) exhibited exceptional photoswitching characteristics. Complexation of the azo group with $BF_2$ leads to $\pi-\pi^*$ transitions in the visible region.[53] By altering the nature of the R group in **13**, isomerization in the far-red and even near-IR was recently achieved.[54] Unfortunately, these species are converted to hydrazones in water, thus limiting their applicability in biological systems. However, modifications to the $BF_2$ groups may change the susceptibility to hydrolysis.

An entirely different approach to driving photoisomerization with long wavelength light is to take advantage of two-photon absorption processes. The two-photon cross section of some bioactive azobenzene-based photoswitches has been analyzed[55] and nonlinear optical responses can be improved by chemical design. For example, **14** includes a naphthalene derivative as an antenna to enhance two-photon absorption.[56] However, these photoswitches can become large and complex, and require the use of femtosecond pulsed laser systems.[57]

## *ortho* SUBSTITUTION

To develop small azo compounds that showed long wavelength photoswitching while maintaining slow (seconds to minutes) thermal relaxation rates in water, we decided to explore introducing amino groups at positions *ortho* to the azo group (Figure 5).[58] Our intent was to introduce electron-donating groups to enhance delocalization, but in a manner that might



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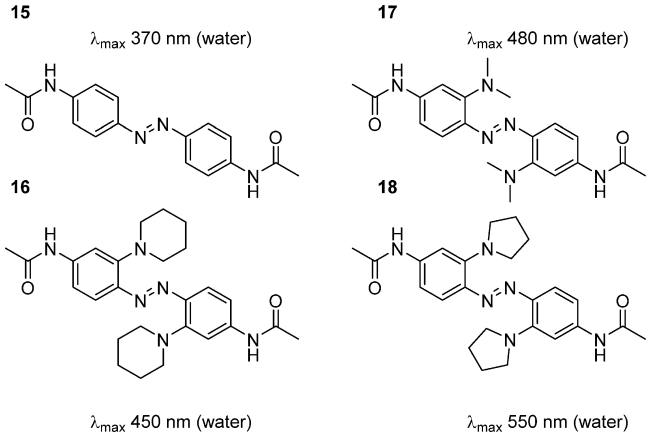


**Figure 5.** Some *ortho*-amino-substituted azobenzenes photoswitches.[58]

affect the steric or electronic barrier for isomerization and thereby slow down thermal relaxation.

This approach worked in the sense that thermal relaxation rates for *ortho* substituted compounds were slower than for corresponding *para* substituted azobenzenes (e.g., half-lives of 100 s vs <1 s).[58] However, as the system became more electron rich, photobleaching was observed. This was not a problem for blue-absorbing (**16**) and cyan-absorbing compounds (**17**), but with the green-absorbing compound **18**, color loss occurred in a few hours under ambient light.

## TETRA-*ortho*-METHOXY SUBSTITUTION

We decided to replace *ortho*-amino groups with methoxy groups with the expectation that the photobleaching observed with *ortho*-amino substituted azobenzenes might be avoided. Since methoxy groups are less electron donating than amino groups, we introduced four rather than two substituents. We made the tetra-*ortho*-methoxy substituted azobenzene (**19**), again with two *p*-amido moieties to permit linkage to target biomolecules.[59] The UV−vis spectrum of the dark-adapted, *trans* isomer is shown in Figure 6. The main $\pi-\pi^*$ transition was blue-shifted and the $n-\pi^*$ transition was red-shifted compared to the parent compound without the methoxy substituents (Figure 6). We proposed that this was due to twisting (nonplanarity) of the *trans* isomer and interaction of the methoxy groups with the N lone pairs on the azo group.[59]

The unusually shifted $n-\pi^*$ transition is only a feature of the *trans* isomer of **19**; the geometry of the *cis* isomer moves the methoxy groups away from the azo nitrogen lone pairs, so the *cis* $n-\pi^*$ transition is less affected by the methoxy groups. As a result, the $n-\pi^*$ transitions of *cis* and *trans* isomers are sufficiently separated to allow *cis*-to-*trans* isomerization with blue light, and *trans*-to-*cis* isomerization with green light, avoiding the use of UV entirely.[59]

A second critical feature of compound **19** is that the thermal back reaction is very slow; the half-life is ∼2 days at room temperature in aqueous solution.[59] Thus, we had discovered a simple derivative that could be switched with green light (like *ortho* amino compound **18** above) did not photobleach, was stable in water, and had a very slow thermal back reaction.

When we attached derivatives of **19** via their *p*-amido groups to a series of peptides the exact position of the $n-\pi^*$ transition of the *trans* isomer was found to depend on the peptide sequence.[35] It seemed that subtle variations in solvation and H-bonding could affect the orientations of the methoxy groups and the degree of twisting and, thereby, the position of the *trans* $n-\pi^*$ band. We noticed that, with some of these peptides, the tail of the $n-\pi^*$ transition extended past 600 nm into the red region of the spectrum; while the absorption coefficient in the red was small, it was not zero (Figure 6). The absorption coefficient of the *cis* isomer was much smaller than that of the *trans*, because its $n-\pi^*$ transition was not shifted. When the sample was irradiated with a high power red-emitting LED we found that almost complete (98%) *trans*-to-*cis* isomerization occurred. The very slow thermal back reaction (hours) at ambient temperature in aqueous solution meant that, even although the rate of *trans*-to-*cis* photoisomerization was slow (due to the inefficient absorption process), effective switching occurred.

We thought we had a photoswitch with most of the characteristics (long wavelength switching, stable in water) needed for in vivo use. We expected, because it was electron rich, **19** should also be stable to reduction by thiols. However, we found the compound was reduced in a few hours in 10 mM glutathione.[35] This meant the photoswitch could only be used in more oxidizing environments (i.e., extracellular environments, or directed to intracellular endoplasmic reticulum/Golgi compartments) in vivo. We then found that replacing the methoxy groups with electron-withdrawing Cl atoms (**20**) (Figure 7) completely circumvented this sensitivity to reduction. The tetra-*ortho*-chloro compound also has red-shifted *trans* $n-\pi^*$ transitions, albeit somewhat less so than **19**.[35] We also found that tetra-*ortho*-thioether groups (**21**) could be used to circumvent reduction while enhancing the molar absorption coefficient in the red region compared to the tetra-*ortho*-chloro species.[36] About the same time, Hecht's

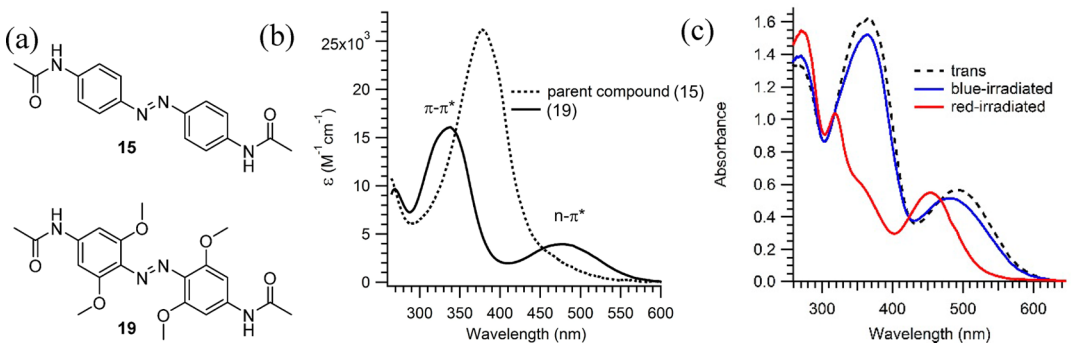


**Figure 6.** (a) Tetra-*ortho*-methoxy substituted bis-*p*-amido azobenzene **19**, and the parent compound **15**. (b) Corresponding UV−vis spectra (trans isomers) in aqueous solution. (c) UV−vis spectra of **19** attached to a peptide in *cis* and *trans* conformations.



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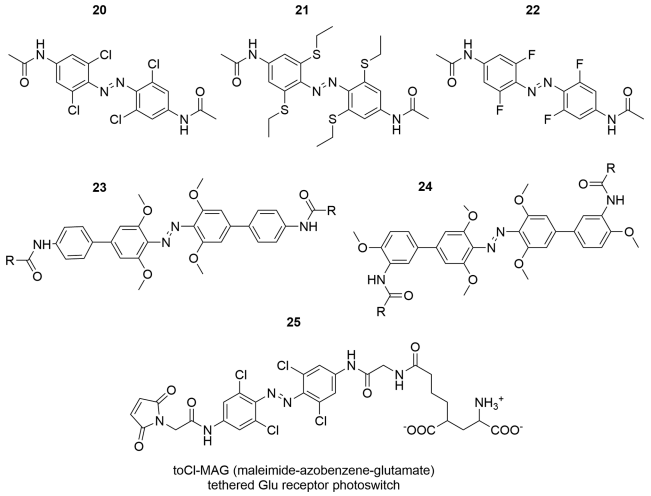


**Figure 7.** Various tetra-*ortho*-substituted azobenzene derivatives. For references, see **17**,[35] **18**,[36] **19**,[60] **20**,[35] **21**, and **22**.[61]

group synthesized the tetra-*ortho*-fluoro substituted species (**22**).[60] While this does not have the same degree of n−$\pi^*$ red shifting, it has an extraordinarily slow thermal *cis*-to-*trans* relaxation rate (years) so that it is truly bistable on biological time scales.[60]

The tetra *ortho* substitution pattern has proven very versatile. We developed extended versions of the photoswitch that show enhanced end-to-end distance changes upon photoswitching (**23**, **24**) and can be used to control peptide conformation.[35] Using Trauner's approach to synthesizing tethered ligands for glutamate receptors, we made tetra-*ortho*-chloro MAG (**25**), which Isacoff's group showed could be used to control Glu receptor activity with red light in living cells.[61]

## TETRA-*ortho*-METHOXY SUBSTITUTED AZONIUM IONS

In the course of making derivatives of the tetra *ortho*-methoxy scaffold, we made the bis-*p*-amino substituted compound **26**, in order to red-shift the spectra further.[62] These compounds were strongly blue colored at neutral pH. The color suggested the presence of an azonium ion, but these typically occur at pH < 3.5 where the azo group becomes protonated. Azonium ions do not usually appear to photoisomerize because the thermal back reaction is very fast (typically microseconds;[63] one needs flash photolysis equipment to detect it). To our surprise, when a solution of **26** was irradiated with red light at pH 7, clear photochromism was observed with a thermal relaxation half-life of seconds, a million times slower than that of a typical azonium ion.

We believe the *ortho* methoxy groups stabilize the azonium ion via resonance but also by providing H-bond acceptors. H-bonding of the azonium proton to the methoxy groups is mainly a feature of the *trans* isomer; the methoxy group is too far away to be a H-bond acceptor in the *cis* isomer. Calculated minimum energy structures of the *cis* and *trans* azonium ions of a simplified model of **26** are shown in Figure 9. Absence of an H-bond in the *cis* isomer decreases its p$K_a$, leading to the production of the neutral *cis* isomer at pH 7 and overall slowing of the thermal relaxation rate.[62]

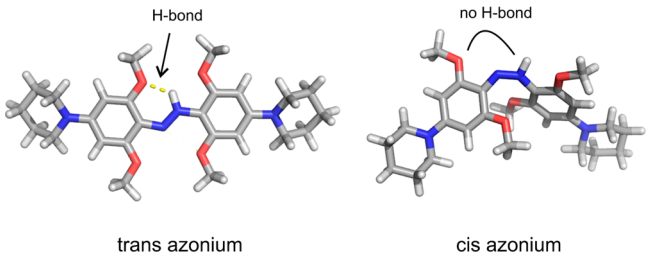


**Figure 9.** Calculated minimum energy structures (B3LYP, 6-311+ +G**) of *trans* and *cis* azonium ions formed by a tetra-*ortho*-methoxy substituted *p*-piperidino azobenzene photoswitch (a model for **26**).

Compound **26** absorbs strongly in the red (the absorption coefficient is ∼100 times larger than that of the amide compound **19** at 630 nm),[64] meaning photoswitching with low intensity red light is fast (seconds). It is water stable and stable to photobleaching over several thousand cycles. It is sensitive to reduction by thiols, so that use in extracellular environments is preferred.[62] Although the thermal barrier to isomerization is low, the *trans* isomer is still substantially more stable than the *cis* isomer. Quantum chemical calculations (B3LYP, 6-311+ +G**) indicate that the trans azonium ion shown in Figure 9 is ∼11 kcal/mol more stable than the cis; for the neutral species, the difference is 8 kcal/mol. Overall the tetra-*ortho*-methoxy

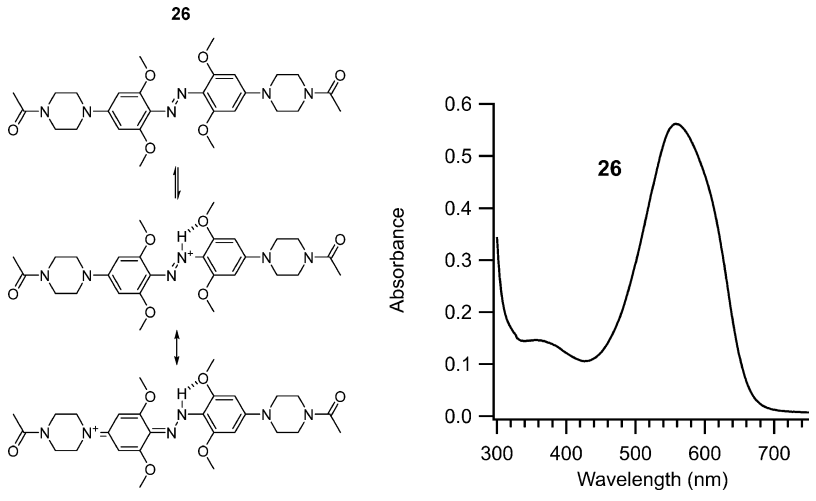


**Figure 8.** Azonium ion formed by a tetra-*ortho*-methoxy substituted *p*-piperazino azobenzene.



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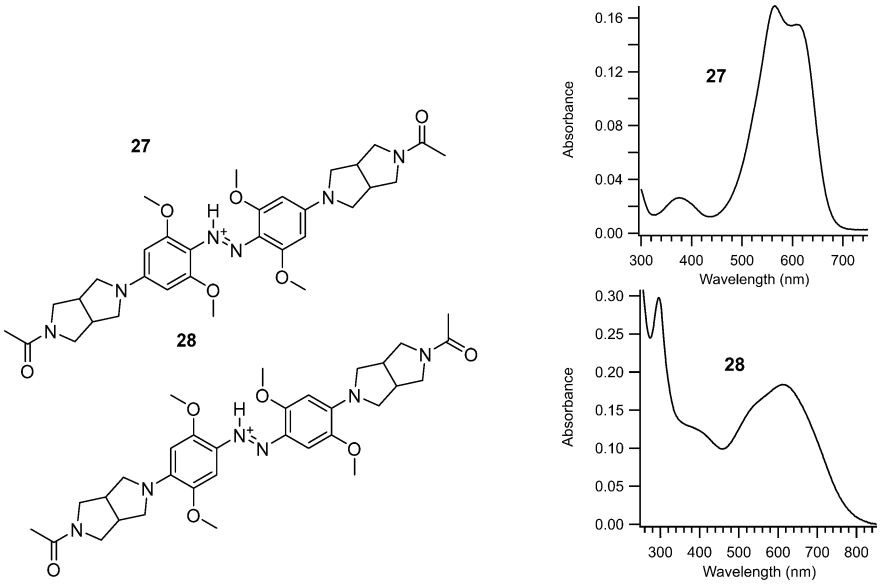


**Figure 10.** Far-red and near-IR absorbing azonium ions and their UV−vis spectra in aqueous solution.[64]

substituted azonium-based photoswitch has a number of very appealing properties. Photoswitching with **26** has been demonstrated in whole blood, confirming its suitability for in vivo use.[62]

## FAR-RED AND NEAR-IR AZONIUM IONS

Encouraged by these observations with **26**, we have been attempting to push the absorbance further into the red and near-IR. We have again been guided by old azobenzene literature, as well as modern quantum chemical calculations. The role of the relative positions of multiple methoxy groups on the color of azo and azonium compounds was studied as far back as 1910 by Kauffmann and Kugel.[65] These studies were systemized and extended by Wizinger in the 1960s and 1970s.[66] His "distribution rules for auxochromes" predict a second methoxy substituent in a *meta* position should shift absorbance to longer wavelengths. We systematically explored effects of multiple methoxy groups, as well as *para* substituents of enhanced electron donating ability, on the behavior of these molecules. This work has led to compounds such as **27** and **28**. Compound **27** with *para*-pyrrolidino groups operates in aqueous solution with far-red light (660 nm) (Figure 10).[64] Compound **28** shows near-IR absorbance at 730 nm (an optimal wavelength for tissue penetration). However, its p$K_a$ is relatively low (5.4), meaning the fraction of the azonium species at neutral pH is small. The thermal back reaction of **28** is also relatively fast ($\tau_{1/2}$ = 10 $\mu$s). We hope that, by combining tetra *ortho* substitutions with *meta* methoxy groups, both near-IR absorption and slow thermal relaxation can be realized in a water-stable, nonphotobleaching azo compound.

## SUMMARY

This Account has focused on our efforts to develop long wavelength azobenzene-based photoswitches that can operate in vivo. We have tried to emphasize the fact that there are thousands of researchers who have contributed to the understanding of azobenzenes over the years from whose work there is much to be learned. At the same time, there is still much to be discovered. Given the current interest in a number of laboratories in trying to make photoswitches that will operate in vivo, and the versatility of the azo functional group, it will be exciting to see what properties can be crafted in the years to come.

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### Notes

The authors declare no competing financial interest.

### Biographies

**Mingxin Dong** was born in Henan province, China. He worked at the Beijing Institute of Biotechnology and completed a Ph.D. in 2010 with Qiuyun Dai. From 2013 to 2015, he worked as a postdoctoral fellow with Prof. Woolley. His research interests are focused on the design and synthesis of long wavelength azobenzenes and their application to biological systems.

**Amirhossein Babalhavaeji** was born in Hamedan, Iran. He obtained his Bachelor's degree in Chemistry from Sharif University of Technology in Tehran. In 2012, he moved to Canada for doctoral studies with Prof. Woolley. His research is focused on the development of light-switchable proteins by using azobenzene-based cross-linkers

**Subhas Samanta** was born in Hooghly, India and received his Ph.D. degree in chemistry from IIT Kanpur. He was a postdoctoral fellow with Prof. Andrew Woolley. Currently, he is a postdoctoral research associate, University of Pittsburgh with Prof. Alexander Deiters. His current research involves genetically encoded light-active unnatural amino acids and small-molecule inhibitors of microRNAs.

**Andrew A. Beharry** was born in Toronto. He obtained a Ph.D. at the University of Toronto where he specialized in azobenzene photoswitches for biological applications. He is currently a Human Frontiers Science Program Scholar at Stanford. His research entails developing DNA-based fluorescent chemosensors for monitoring the activity of DNA repair enzymes.

**G. Andrew (Drew) Woolley** was born in Edinburgh, Scotland, but at a young age he moved to Toronto. He attended the University of Toronto, completing a B.Sc. in Chemistry and a Ph.D. in Biochemistry



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with Charles Deber. After postdoctoral stints with Bonnie Wallace in New York and London, he returned to Toronto to join the Chemistry Department where he is now Professor. Drew won the Rutherford Medal from The Royal Society of Canada for his work on peptide and protein design. He is especially interested in the design of photoswitchable proteins.

## REFERENCES


(1) Hartley, G. S. The cis-form of azobenzene. *Nature* **1937**, *140*, 281−281.
(2) Rau, H.: Azo Compounds. In *Photochromism, Molecules and Systems*; Durr, H., Bouas-Laurent, H., Eds.; Elsevier: Amsterdam, 2003; pp 165−192.
(3) Beharry, A. A.; Woolley, G. A. Azobenzene photoswitches for biomolecules. *Chem. Soc. Rev.* **2011**, *40*, 4422−4437.
(4) Kaufman, H.; Vratsanos, S. M.; Erlanger, B. F. Photoregulation of an enzymic process by means of a light-sensitive ligand. *Science* **1968**, *162*, 1487−1489.
(5) Bartels, E.; Wassermann, N. H.; Erlanger, B. F. Photochromic activators of the acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **1971**, *68*, 1820−1823.
(6) Erlanger, B. F.; Wassermann, N. H.; Cooper, A. G.; Monk, R. J. Allosteric activation of the hydrolysis of specific substrates by chymotrypsin. *Eur. J. Biochem.* **1976**, *61*, 287−295.
(7) Gurney, A. M.; Lester, H. A. Light-flash physiology with synthetic photosensitive compounds. *Physiol Rev.* **1987**, *67*, 583−617.
(8) Wyart, C.; Del Bene, F.; Warp, E.; Scott, E. K.; Trauner, D.; Baier, H.; Isacoff, E. Y. Optogenetic dissection of a behavioural module in the vertebrate spinal cord. *Nature* **2009**, *461*, 407−410.
(9) Banghart, M.; Borges, K.; Isacoff, E.; Trauner, D.; Kramer, R. H. Light-activated ion channels for remote control of neuronal firing. *Nat. Neurosci.* **2004**, *7*, 1381−1386.
(10) Bose, M.; Groff, D.; Xie, J.; Brustad, E.; Schultz, P. G. The incorporation of a photoisomerizable amino acid into proteins in E. coli. *J. Am. Chem. Soc.* **2006**, *128*, 388−389.
(11) Szymanski, W.; Beierle, J. M.; Kistemaker, H. A.; Velema, W. A.; Feringa, B. L. Reversible photocontrol of biological systems by the incorporation of molecular photoswitches. *Chem. Rev.* **2013**, *113*, 6114−6178.
(12) Tsai, Y. H.; Essig, S.; James, J. R.; Lang, K.; Chin, J. W. Selective, rapid and optically switchable regulation of protein function in live mammalian cells. *Nat. Chem.* **2015**, *7*, 554−561.
(13) Volgraf, M.; Gorostiza, P.; Numano, R.; Kramer, R. H.; Isacoff, E. Y.; Trauner, D. Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat. Chem. Biol.* **2006**, *2*, 47−52.
(14) Levitz, J.; Pantoja, C.; Gaub, B.; Janovjak, H.; Reiner, A.; Hoagland, A.; Schoppik, D.; Kane, B.; Stawski, P.; Schier, A. F.; Trauner, D.; Isacoff, E. Y. Optical control of metabotropic glutamate receptors. *Nat. Neurosci.* **2013**, *16*, 507−516.
(15) Tochitsky, I.; Banghart, M. R.; Mourot, A.; Yao, J. Z.; Gaub, B.; Kramer, R. H.; Trauner, D. Optochemical control of genetically engineered neuronal nicotinic acetylcholine receptors. *Nat. Chem.* **2012**, *4*, 105−111.
(16) Lin, W. C.; Davenport, C. M.; Mourot, A.; Vytla, D.; Smith, C. M.; Medeiros, K. A.; Chambers, J. J.; Kramer, R. H. Engineering a light-regulated GABAA receptor for optical control of neural inhibition. *ACS Chem. Biol.* **2014**, *9*, 1414−1419.
(17) Kumar, K. R.; Kamei, T.; Fukaminato, T.; Tamaoki, N. Complete ON/OFF photoswitching of the motility of a nano-biomolecular machine. *ACS Nano* **2014**, *8*, 4157−4165.
(18) Ali, A. M.; Forbes, M. W.; Woolley, G. A. Optimizing the photocontrol of bZIP coiled coils with azobenzene crosslinkers: Role of the crosslinking site. *ChemBioChem* **2015**, *16*, 1757.
(19) Pittolo, S.; Gomez-Santacana, X.; Eckelt, K.; Rovira, X.; Dalton, J.; Goudet, C.; Pin, J. P.; Llobet, A.; Giraldo, J.; Llebaria, A.; Gorostiza, P. An allosteric modulator to control endogenous G protein-coupled receptors with light. *Nat. Chem. Biol.* **2014**, *10*, 813−815.
(20) Broichhagen, J.; Frank, J. A.; Trauner, D. A roadmap to success in photopharmacology. *Acc. Chem. Res.* **2015**, *48*, 1947−1960.
(21) Velema, W. A.; Szymanski, W.; Feringa, B. L. Photopharmacology: beyond proof of principle. *J. Am. Chem. Soc.* **2014**, *136*, 2178−2191.
(22) Stein, M.; Middendorp, S. J.; Carta, V.; Pejo, E.; Raines, D. E.; Forman, S. A.; Sigel, E.; Trauner, D. Azo-propofols: photochromic potentiators of GABA(A) receptors. *Angew. Chem., Int. Ed.* **2012**, *51*, 10500−10504.
(23) Schonberger, M.; Trauner, D. A photochromic agonist for mu-opioid receptors. *Angew. Chem., Int. Ed.* **2014**, *53*, 3264−3267.
(24) Velema, W. A.; van der Berg, J. P.; Hansen, M. J.; Szymanski, W.; Driessen, A. J.; Feringa, B. L. Optical control of antibacterial activity. *Nat. Chem.* **2013**, *5*, 924−928.
(25) Broichhagen, J.; Frank, J. A.; Johnston, N. R.; Mitchell, R. K.; Smid, K.; Marchetti, P.; Bugliani, M.; Rutter, G. A.; Trauner, D.; Hodson, D. J. A red-shifted photochromic sulfonylurea for the remote control of pancreatic beta cell function. *Chem. Commun.* **2015**, *51*, 6018−6021.
(26) Jones, D. E. H. Ariadne. *New Sci.* **1976**, *70*, 560.
(27) Borowiak, M.; Nahaboo, W.; Reynders, M.; Nekolla, K.; Jalinot, P.; Hasserodt, J.; Rehberg, M.; Delattre, M.; Zahler, S.; Vollmar, A.; Trauner, D.; Thorn-Seshold, O. Photoswitchable inhibitors of microtubule dynamics optically control mitosis and cell death. *Cell* **2015**, *162*, 403−411.
(28) Tochitsky, I.; Polosukhina, A.; Degtyar, V. E.; Gallerani, N.; Smith, C. M.; Friedman, A.; Van Gelder, R. N.; Trauner, D.; Kaufer, D.; Kramer, R. H. Restoring visual function to blind mice with a photoswitch that exploits electrophysiological remodeling of retinal ganglion cells. *Neuron* **2014**, *81*, 800−813.
(29) Bellotto, S.; Chen, S.; Rentero Rebollo, I.; Wegner, H. A.; Heinis, C. Phage selection of photoswitchable peptide ligands. *J. Am. Chem. Soc.* **2014**, *136*, 5880−5883.
(30) Jafari, M. R.; Deng, L.; Kitov, P. I.; Ng, S.; Matochko, W. L.; Tjhung, K. F.; Zeberoff, A.; Elias, A.; Klassen, J. S.; Derda, R. Discovery of light-responsive ligands through screening of a light-responsive genetically encoded library. *ACS Chem. Biol.* **2014**, *9*, 443−450.
(31) Lesch, J. E.: *The first miracle drugs: How the sulfa drugs transformed medicine*; Oxford University Press: New York, 2007.
(32) Thomas, B. H.; Whitehouse, L. W.; Solomonraj, G.; Paul, C. J. Excretion of phenazopyridine and its metabolites in the urine of humans, rats, mice, and guinea pigs. *J. Pharm. Sci.* **1990**, *79*, 321−325.
(33) Brown, M. A.; Devito, S. C. Predicting Azo-Dye Toxicity. *Crit. Rev. Environ. Sci. Technol.* **1993**, *23*, 249−324.
(34) Beharry, A. A.; Wong, L.; Tropepe, V.; Woolley, G. A. Fluorescence imaging of azobenzene photoswitching in vivo. *Angew. Chem., Int. Ed.* **2011**, *50*, 1325−1327.
(35) Samanta, S.; Beharry, A. A.; Sadovski, O.; McCormick, T. M.; Babalhavaeji, A.; Tropepe, V.; Woolley, G. A. Photoswitching azo compounds in vivo with red light. *J. Am. Chem. Soc.* **2013**, *135*, 9777−9784.
(36) Samanta, S.; McCormick, T. M.; Schmidt, S. K.; Seferos, D. S.; Woolley, G. A. Robust visible light photoswitching with ortho-thiol substituted azobenzenes. *Chem. Commun.* **2013**, *49*, 10314−10316.
(37) Heckel, A.; Mayer, G.: Light-responsive nucleic acids for the spatiotemporal control of biological processes. In *The chemical biology of nucleic acids*; Mayer, G., Ed.; Wiley: Chichester, 2010; pp 279−306.
(38) Cheong, W. F.; Prahl, S. A.; Welch, A. J. A Review of the Optical-Properties of Biological Tissues. *IEEE J. Quantum Electron.* **1990**, *26*, 2166−2185.
(39) Bléger, D.; Hecht, S. Visible-light-activated molecular switches. *Angew. Chem., Int. Ed.* **2015**, *54*, 11338.
(40) Garcia-Amoros, J.; Velasco, D. Recent advances towards azobenzene-based light-driven real-time information-transmitting materials. *Beilstein J. Org. Chem.* **2012**, *8*, 1003−1017.
(41) Bello, K. A.; Griffiths, J. Azo dyes with absorption bands in the near-infrared. *J. Chem. Soc., Chem. Commun.* **1986**, 1639−1640.



DOI: 10.1021/acs.accounts.5b00270
*Acc. Chem. Res.* 2015, 48, 2662−2670

(42) Li, Y.; Patrick, B. O.; Dolphin, D. Near-infrared absorbing azo dyes: Synthesis and X-ray crystallographic and spectral characterization of monoazopyrroles, bisazopyrroles, and a boron-azopyrrole complex. *J. Org. Chem.* **2009**, *74*, 5237−5243.
(43) Salvador, M. A.; Almeida, P.; Reis, L. V.; Santos, P. F. Near-infrared absorbing delocalized cationic azo dyes. *Dyes Pigm.* **2009**, *82*, 118−123.
(44) Garcia-Amoros, J.; Diaz-Lobo, M.; Nonell, S.; Velasco, D. Fastest Thermal Isomerization of an Azobenzene for Nanosecond Photoswitching Applications under Physiological Conditions. *Angew. Chem., Int. Ed.* **2012**, *51*, 12820−12823.
(45) Burns, D. C.; Flint, D. G.; Kumita, J. R.; Feldman, H. J.; Serrano, L.; Zhang, Z.; Smart, O. S.; Woolley, G. A. Origins of helix-coil switching in a light-sensitive peptide. *Biochemistry* **2004**, *43*, 15329−15338.
(46) Beharry, A. A.; Chen, T.; Al-Abdul-Wahid, M. S.; Samanta, S.; Davidov, K.; Sadovski, O.; Ali, A. M.; Chen, S. B.; Prosser, R. S.; Chan, H. S.; Woolley, G. A. Quantitative analysis of the effects of photoswitchable distance constraints on the structure of a globular protein. *Biochemistry* **2012**, *51*, 6421−6431.
(47) Siewertsen, R.; Neumann, H.; Buchheim-Stehn, B.; Herges, R.; Nather, C.; Renth, F.; Temps, F. Highly efficient reversible Z-E photoisomerization of a bridged azobenzene with visible light through resolved S(1)(n pi*) absorption bands. *J. Am. Chem. Soc.* **2009**, *131*, 15594−15595.
(48) Samanta, S.; Qin, C. G.; Lough, A. J.; Woolley, G. A. Bidirectional photocontrol of peptide conformation with a bridged azobenzene derivative. *Angew. Chem., Int. Ed.* **2012**, *51*, 6452−6455.
(49) Sell, H.; Nather, C.; Herges, R. Amino-substituted diazocines as pincer-type photochromic switches. *Beilstein J. Org. Chem.* **2013**, *9*, 1−7.
(50) Tellkamp, T.; Shen, J.; Okamoto, Y.; Herges, R. Diazocines on Molecular Platforms. *Eur. J. Org. Chem.* **2014**, *2014*, 5456−5461.
(51) Joshi, D. K.; Mitchell, M. J.; Bruce, D.; Lough, A. J.; Yan, H. B. Synthesis of cyclic azobenzene analogues. *Tetrahedron* **2012**, *68*, 8670−8676.
(52) Weston, C. E.; Richardson, R. D.; Haycock, P. R.; White, A. J.; Fuchter, M. J. Arylazopyrazoles: azoheteroarene photoswitches offering quantitative isomerization and long thermal half-lives. *J. Am. Chem. Soc.* **2014**, *136*, 11878−11881.
(53) Yang, Y.; Hughes, R. P.; Aprahamian, I. Visible light switching of a BF2-coordinated azo compound. *J. Am. Chem. Soc.* **2012**, *134*, 15221−15224.
(54) Yang, Y.; Hughes, R. P.; Aprahamian, I. Near-infrared light activated azo-BF2 switches. *J. Am. Chem. Soc.* **2014**, *136*, 13190−13193.
(55) Carroll, E. C.; Berlin, S.; Levitz, J.; Kienzler, M. A.; Yuan, Z.; Madsen, D.; Larsen, D. S.; Isacoff, E. Y. Two-photon brightness of azobenzene photoswitches designed for glutamate receptor optogenetics. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E776−785.
(56) Izquierdo-Serra, M.; Gascon-Moya, M.; Hirtz, J. J.; Pittolo, S.; Poskanzer, K. E.; Ferrer, E.; Alibes, R.; Busque, F.; Yuste, R.; Hernando, J.; Gorostiza, P. Two-photon neuronal and astrocytic stimulation with azobenzene-based photoswitches. *J. Am. Chem. Soc.* **2014**, *136*, 8693−8701.
(57) Sakadzic, S.; Demirbas, U.; Mempel, T. R.; Moore, A.; Ruvinskaya, S.; Boas, D. A.; Sennaroglu, A.; Kaertner, F. X.; Fujimoto, J. G. Multi-photon microscopy with a low-cost and highly efficient Cr:LiCAF laser. *Opt. Express* **2008**, *16*, 20848−20863.
(58) Sadovski, O.; Beharry, A. A.; Zhang, F.; Woolley, G. A. Spectral tuning of azobenzene photoswitches for biological applications. *Angew. Chem., Int. Ed.* **2009**, *48*, 1484−1486.
(59) Beharry, A. A.; Sadovski, O.; Woolley, G. A. Azobenzene photoswitching without ultraviolet light. *J. Am. Chem. Soc.* **2011**, *133*, 19684−19687.
(60) Bleger, D.; Schwarz, J.; Brouwer, A. M.; Hecht, S. o-Fluoroazobenzenes as readily synthesized photoswitches offering nearly quantitative two-way isomerization with visible light. *J. Am. Chem. Soc.* **2012**, *134*, 20597−20600.
(61) Rullo, A.; Reiner, A.; Reiter, A.; Trauner, D.; Isacoff, E. Y.; Woolley, G. A. Long wavelength optical control of glutamate receptor ion channels using a tetra-ortho-substituted azobenzene derivative. *Chem. Commun.* **2014**, *50*, 14613−14615.
(62) Samanta, S.; Babalhavaeji, A.; Dong, M. X.; Woolley, G. A. Photoswitching of ortho-substituted azonium ions by red light in whole blood. *Angew. Chem., Int. Ed.* **2013**, *52*, 14127−14130.
(63) Sanchez, A. M.; Barra, M.; de Rossi, R. H. On the mechanism of the acid/base-catalyzed thermal cis-trans isomerization of methyl orange. *J. Org. Chem.* **1999**, *64*, 1604−1609.
(64) Dong, M.; Babalhavaeji, A.; Hansen, M. J.; Kalman, L.; Woolley, G. A. Red, far-red, and near infrared photoswitches based on azonium ions. *Chem. Commun.* **2015**, *51*, 12981−12984.
(65) Kauffmann, H.; Kugel, W. A proposition of distribution of auxochromes and azo compounds. *Ber. Dtsch. Chem. Ges.* **1911**, *44*, 2386−2389.
(66) Wizinger, R. Der Verteilungssatz Der Auxochrome (Distribution rule of auxochromes). *Chimia* **1965**, *19*, 339−350.


DOI: 10.1021/acs.accounts.5b00270
*Acc. Chem. Res.* 2015, 48, 2662−2670