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Red, far-red, and near infrared photoswitches based on azonium ions†

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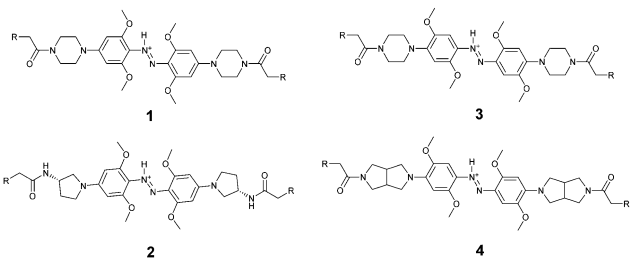
Azonium ions formed by *p*-amino substituted azo compounds with both *ortho*- and *meta*-methoxy substituents exhibit strong absorbance in far-red and near infrared spectral region. The compounds undergo robust photoswitching in aqueous solution and exhibit a range of thermal relaxation rates from 10 μs–100 ms.

Molecules that undergo reversible light driven changes in structure are of increasing interest for applications in fields ranging from molecular electronics to neurobiology.[1–3] For biological applications in particular, considerable efforts have been made to produce molecules that absorb in the red region of the visible spectrum since these wavelengths enable much greater tissue penetration and less phototoxicity than shorter wavelengths.[4–9] Ideally, however, molecular photoswitches designed for *in vivo* use should operate in the near infrared (IR) optical window.[6,10] The edges of the near IR window depend on the tissue in question, but are generally set by haemoglobin absorption at shorter wavelengths and water absorption at longer wavelengths. The best penetration is often observed near 730 nm.[11] Although some near IR absorbing azo compounds are known, these are complex, flexible, molecules that would be difficult to apply as photoswitches.[12,13] Very recently Aprahamian and colleagues described near IR absorbing photoswitches based on BF_2-modified azo compounds.[10] These compounds showed excellent photochemical characteristics, however, their tendency to hydrolyse in aqueous solution presents a serious drawback to their application in a biological context.

We recently described the azonium ion **1** that absorbed red light and underwent *trans–cis* photoisomerization.[14] It relaxed to the *trans* isomer in the dark on the timescale of seconds so that pulses of red light could be used to drive multiple isomerization cycles. Resonance stabilization of the azonium cation together with intramolecular H-bonding between the azonium proton and methoxy groups *ortho* to the azo unit meant that the *trans* azonium species was present at pH 7 in aqueous solution, whereas typically azonium ions are only formed from aminoazobenzenes at pH < 3.5.[15–17] The geometry of the *cis* isomer prevents formation of this intramolecular H-bond so that the *cis* azonium pK_a is lower than that of the *trans* isomer.[14] As a result, depending on the pH, *trans* to *cis* isomerization is accompanied by proton dissociation to produce the neutral *cis* species, thereby slowing thermal reversion to the *trans* form. This feature is useful for the photochemical production of a large fraction of this *cis* isomer to control molecular targets. Since compound **1** acts as an effective red light switch that can operate in biological milieu, we used it as a starting point to try to design azonium ion based switches that would operate in the near IR.



There is a rich literature describing substituent effects on the absorption wavelengths of azobenzenes and, to a lesser extent, the corresponding azonium ions.[18] We wished to keep at least one methoxy substituent in an *ortho* position to preserve the possibility of intramolecular H-bonding of the *trans* azonium ion. We therefore considered changes at *para* and *meta* positions. Varying the nature of the amine in the *para* position alters the degree to which the *p*-amino nitrogen lone pairs delocalize into the ring system.[19] A second phenomenon, called the distribution rule of auxochromes was recognized early by Kaufmann[20] and described in detail by Wizinger.[21] This rule predicts that a second auxochrome (*e.g.* a methoxy substituent)

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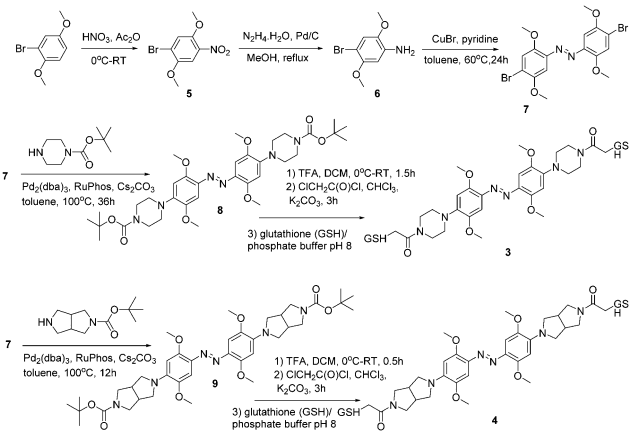
in a *meta* position should shift absorbance to longer wavelengths. The phenomenon is not explained using heuristic rules of resonance and historically was the subject of much discussion,[21,22] but nowadays is predicted by standard quantum chemical calculations. In an effort to produce photoswitches operating at longer wavelengths than (**1**), we decided to examine the role of the methoxy substitution pattern as well as the nature of the *para* amino group in the photochemical behaviour of these azonium-based photoswitches.

Compounds **2**, **3**, and **4** were designed to preserve the possibility of H-bonding of the azonium proton to an *ortho*-methoxy group in the *trans* isomer (as in **1**) but to have longer wavelength absorption due to the presence of better *para* donating substituents (**2** *vs.* **1**, **4** *vs.* **3**) or an *ortho–meta* methoxy substitution pattern (**3** and **4** *vs.* **1** and **2**). In addition to affecting the wavelength of absorption, these changes are expected to alter the pK_a of the azonium ion formed in each case. Calculations were performed using density functional methods (B3LYP (6-31++G**)) to optimize geometry and TDDFT to calculate absorption wavelength maxima (see ESI†). These computational methods have been used successfully with related compounds.[23,24] TDDFT calculations predict that these compounds should absorb at longer wavelengths than **1** (Table 1). Calculating effects of the substitution patterns on pK_as is problematic[25] and was not attempted here.

Compound **2** was synthesized in a manner analogous to that reported previously for **1** except that an amino-pyrrolidine unit replaced the piperazine unit in **1** (see ESI† for details). Compounds **3** and **4** were synthesized as outlined in Scheme 1. Commercially available 1-bromo-2,5-dimethoxybenzene was nitrated to give compound **5**. Reduction using hydrazine hydrate and Pd/C, produced compound **6**. The key intermediate **7** was generated by treating **6** with CuBr and pyridine in toluene.

To produce **4** we used a bicyclic pyrrolidine to preserve the symmetry of the compound and to maximize the end-to-end distance change upon *trans* to *cis* isomerization; both these features are important for applying these compounds as cross-linkers for peptides and proteins.[26] The bicyclic pyrrolidine version of **2** proved to be insoluble so that it was replaced by the monocyclic amino-pyrrolidine in that case. These different pyrrolidine moieties are expected to behave similarly in terms of electron donation to the azo system. In each case, the compound was linked at both ends to the short tripeptide glutathione (GSH). This confers water solubility on the photoswitch and also provides a test of the operational sensitivity of the compound to reduction by thiols. While azonium ions can, in general, be reduced by thiols[14,24] the rate of reduction



Scheme 1

depends on the pH and thiol concentration which vary widely depending on the particular biological environment in question.[27] In practice, we find that if a GSH adduct can be readily synthesized, the rate of reduction is slow enough that the compound can be used under typical physiological conditions.

The absorption properties of compounds **2**, **3**, and **4** were examined in aqueous buffer as a function of pH. These compounds were found to be stable for weeks at room temperature in aqueous solutions near neutral pH, an important requirement for biological applications. Fig. 1 shows UV-Vis absorption spectra for each newly synthesized compound together with those obtained previously for compound **1**. In the high pH limit, the spectrum in each case is assumed to correspond to that of the neutral (unprotonated) species. As the pH is lowered, a species with long wavelength absorption appears and this is assumed to be the singly protonated azonium ion. Contributions to the spectra from doubly protonated species (azonium plus ammonium) are indicated by dashed grey lines in Fig. 1 where these dominate the spectra. Apparent values for the pK_as of the azonium ions are indicated in each case, obtained by fitting the observed spectra as function of pH (see the ESI†). The molar extinction coefficients of the *trans* azonium forms of **1–4** are collected in Table 1 (see ESI† for methods). These are similar to that found for methyl orange (55 000 M^{-1} cm^{-1}).[28] TDDFT calculations also predict large oscillator strengths for these compounds (see ESI†).

Increasing the donating ability of the *para* amino substituent moves absorption from the red region into the far-red (compound **2** *vs.* **1**). Introducing the *ortho–meta* substitution pattern produces azonium ions absorbing in the far-red (**3**) and near IR (**4**). Compound **4** shows significant absorbance in the near IR window at 730 nm.

Table 1 Predicted (B3LYP (6-31++G**)) and observed properties of compounds **1–4**

Compound	Predicted λ_{max} (*trans* neutral form) (nm)	Observed λ_{max} (*trans* neutral form) (nm)	Predicted λ_{max} (*trans* azonium form) (nm)	Observed λ_{max} (*trans* azonium form) (nm)	ε (λ_{max}) (*trans* azonium form) (M^{-1} cm^{-1})	Apparent pK_a of azonium ion
1	522, 384	460, 365	545	563	47 000	7.5
2	518, 384	491, 410	544	610	51 000	8.9
3	497	490	631	630	40 000	2.6
4	485	517	634	670	37 000	5.4

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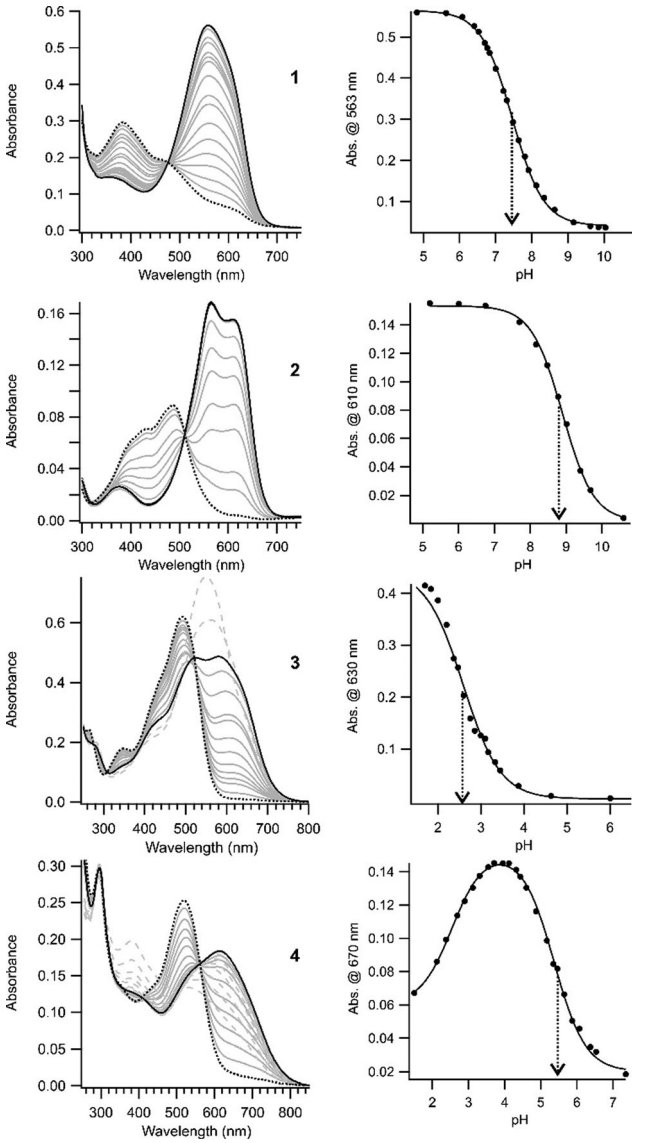

Fig. 1 (left) UV-Vis spectra of the *trans* forms of compounds **1–4** (linked to GSH) in aqueous buffer as a function of pH. In each case, the spectrum at the high pH limit is shown as a black dotted line. Lowering the pH produces the azonium ion (shown as a series of grey solid lines) until a maximum amount of the azonium ion is obtained (solid black line). Further decreases to the pH produce doubly protonated species (azonium plus ammonium). The spectra of these species are indicated by dashed grey lines. (right) pH dependencies of the azonium ions formed in each compound. Solid lines represent the fits to eqn (1) for compounds **1**, **2**, and **3**, and eqn (2) for compound **4**. The dotted arrows mark the pK_as of the azonium ions (see ESI†).

Whether the azonium ion is present at physiological pHs near ~7, depends on the pK_a. The apparent pK_as of these compounds are collected in Table 1. Increasing the donating ability of the *para* amino substituent increased the pK_a of the *trans* azonium ion of 2 to 8.9 (compared to 7.5 for **1**). A consequence of this change is that the azonium ion dominates at physiological pHs near ~7 so that red and far-red wavelengths are absorbed strongly. Replacing two *ortho* methoxy groups with an *ortho–meta* substitution pattern (*e.g.* **3** *vs.* **1** or **4** *vs.* **2**) causes a significant drop in the pK_a of the corresponding *trans* azonium ions, particularly in the case of **3**. The removal of an *ortho* methoxy group removes a possible resonance contributor for stabilization of the positive charge. In addition, it removes a potential H-bond acceptor; however, calculations suggest that H-bonded species still predominate (see ESI†) suggesting the latter is not a major reason for the drop in the azonium pK_a. Instead, the pK_a drop may result from the presence of *meta* methoxy group which forces the *para* piperazino substituent of 3 to rotate so that the *para* piperazino nitrogen atom is significantly less able to act as an electron donor. Calculated structures of **3** (Fig. S1, ESI†) confirm that the piperazino units are highly twisted. Consistent with this proposal, the less sterically demanding pyrrolidino substituent (compound **4**) shows a significantly higher pK_a (Table 1).

We then examined the photoswitching behaviour of this series of compounds using laser flash photolysis techniques. Hundreds of rounds of *trans–cis* photoisomerization occurred in each case and photocyclization of the type observed with simple azonium ions by Lewis[29] and discussed by Mallory[30] does not seem to occur with these compounds. The rate of *cis* to *trans* thermal relaxation increases as the pH is lowered because *cis* azonium species relax significantly more quickly than their neutral counterparts.[31] Fig. 2 shows the observed first order rate constants for thermal *cis*-to-*trans* relaxation as a function of pH for compounds **1–4** in aqueous solution. In general, the rate constants are bounded at the low pH end by the intrinsic relaxation rate of the *cis* azonium ion and at the high pH end by the intrinsic relaxation rate of the neutral species in each case. Not all pH/rate combinations were experimentally accessible. At a given pH the observed rate reflects both the intrinsic thermal relaxation rate as well as the pK_a of the *cis* azonium ion.[14] At physiological pH (~7) the tetra-*ortho* methoxy substituted species **1** and **2** show *cis* lifetimes on the order of 1 s and ~100 ms respectively.

Compound **2** thus functions as a far-red photoswitch that operates at physiological pHs. Compounds **3** and **4** have lifetimes of ~1 ms and 10 μs respectively at pH 7, however only compound **4** has significant near IR absorbance at this pH. Compound **4** could thus function as a near IR switch with rapid (10 μs) thermal relaxation. While rapid thermal relaxation is ideal for certain applications[32,33] slower relaxation permits substantial fractions of the *cis* isomer to be produced at low irradiation powers and is useful when a large degree of photocontrol of single target biomolecules is desired.[1] The thermal relaxation data make it clear that substitution at all four *ortho* positions is necessary to substantially slow the thermal back reaction. Thus, if a compound with near IR absorbance together with a slow thermal relaxation rate is desired, our data predict that substitution of all four *ortho* positions in addition to a *meta* position will be required.

Substituted azonium ions can function as water-stable photoswitches that operate with red, far-red and near IR light. Whereas tetra-*ortho* methoxy substitution is required for slow thermal reversion, a *meta* methoxy substitution pattern enhances the long wavelength absorbance of azonium ions. A *meta* methoxy

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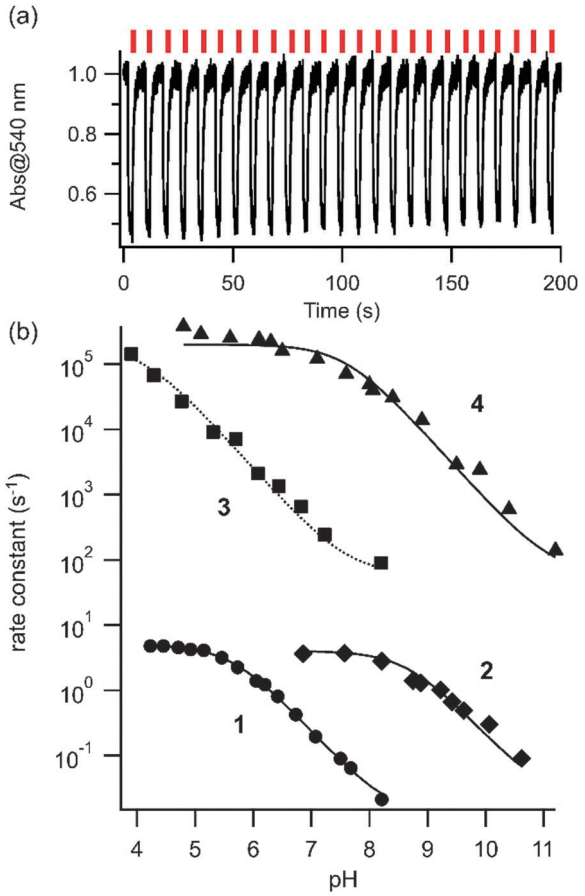

Fig. 2 (a) Far-red photoisomerization of **2**. Absorbance changes observed with pulses of 660 nm light (aqueous buffer, pH 8.8, 20 °C). (b) Observed rate constants for thermal *cis*-to-*trans* relaxation of compounds **1–4** as a function of pH in aqueous buffer. The kinetic traces of thermal relaxation were fit to single exponential decay functions to obtain rate constants (see ESI†). Circles, diamonds, squares, and triangles show the observed rate constants for compounds, **1**, **2**, **3**, and **4**, respectively. The lines represent the fit to eqn (3) yielding pK_a values of the *cis* azonium ions of 5.7, 8.6, 4.1, and 7.6 respectively (see ESI† for details).

substitution places steric constraints on the nature of the *para* substituent so that a pyrrolidino group is best able to act as an electron donor in this position. Varying these substituents allow the construction of azonium-based photoswitches with different degrees of far-red absorbance, thermal relaxation rate and pK_a tuned for desired applications.

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