Article

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Near-infrared photoswitching of azobenzenes under physiological conditions

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ABSTRACT: Biological tissue exhibits an absorbance minimum in the near-infrared between 700-900 nm that permits deep penetration of light. Molecules that undergo photoisomerization in this bio-optical window are highly desirable as core structures for the development of photopharmaceuticals and as components of chemical-biological tools. We report the systematic design, synthesis, and testing of an azobenzene derivative tailored to undergo single-photon photoswitching with near-infrared light under physiological conditions. A fused dioxane ring and a methoxy substituent were used to place oxygen atoms in all four *ortho* positions, as well as two *meta* positions, relative to the azobenzene N=N double bond. This substitution pattern, together with a *para* pyrrolidine group raises the pK_a of the molecule so that it is protonated at physiological pH and absorbs at wavelengths >700 nm. This azobenzene derivative, termed DOM-azo, is stable for months in neutral aqueous solutions, undergoes *trans*-to-*cis* photoswitching with 720 nm light, and thermally reverts to the stable *trans* isomer with a half-life near 1 s.

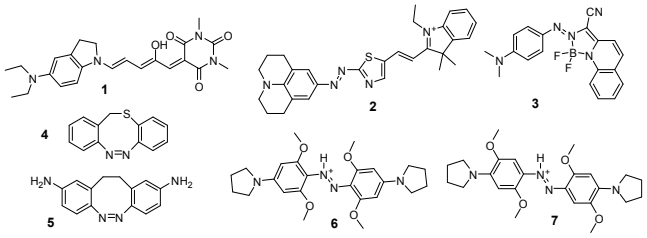
INTRODUCTION

Photoswitching between different conformational states of a molecule provides an exquisite means for external spatial and temporal control of molecular function. This fact is of central importance for the nascent field of photopharmacology in which the action of a photoswitchable bioactive molecule is spatially restricted to desired target areas and can be temporally modulated. Photoswitchable bioactive molecules have now been developed that modulate retinal responses, glucose homeostasis, ion channel function, and the activity of G-protein coupled receptors.[1-3]

For applications *in vivo*, photoswitching in the bio-optical window is desirable, *i.e.* at wavelengths between 700 and 900 nm, to enable effective tissue penetration without the need for fiber optics. This can be achieved using two-photon photoswitching but requires specialized high intensity light sources and molecules with built-in antennae.[4,5] Upconverting nanoparticles provide another means to drive isomerization using multiple, long-wavelength photons[6,7] but the size of nanoparticles places constraints on their applications.[8] It would therefore be highly desirable to identify a molecular structure that undergoes single photon photoswitching with near-infrared (near-IR) light under physiological conditions (*i.e.* aqueous solutions at neutral pH).

Among organic chromophores, donor-acceptor Stenhouse adducts (e.g. **1**) can be tuned to absorb in near-IR ranges[9] but show complex solvent dependent photochemistry that has so far prevented effective photoswitching in water.[10] A variety of azobenzene derivatives show absorbance in the near-IR region (e.g. **2**)[11] but undergo very rapid (<μs) thermal back isomerization meaning that the *cis* isomer content is vanishingly small unless very bright light sources are used.[12] BF_2-adducts of azobenzenes (e.g. **3**) absorb in the near-IR and have slower thermal relaxation (seconds) but hydrolyze in water.[13] Bridged azobenzenes (e.g. **4**, **5**) absorb near-IR wavelengths to produce the thermally more stable isomer.[12,14] For photopharmacological applications *in vivo*, production of the thermally less stable isomer by near-IR light is desirable, otherwise thermal relaxation will lead to bioactivity outside the zone of irradiation. The rate of the thermal back reaction to the stable isomer should be slow enough to permit accumulation of the *cis* isomer under constant near-IR illumination, but fast enough that spatial targeting is not lost. Based on MRI and PET measurements of mean *trans*-it times for blood in the cerebral vasculature, for example,

the appropriate time constant for thermal relaxation should be in the range of 0.1-10 s.[15,16]



Here we present the systematic design, synthesis, and testing of an azobenzene derivative that is tailored to undergo single photon photoswitching with near-IR light under physiological conditions and that thermally reverts to the stable *trans* isomer with a half-life near 1 s. The design was based on extensive azobenzene literature, previous studies on substituted azobenzenes that form azonium ions, as well as computational modeling.

RESULTS and DISCUSSION

Previous work on azobenzene derivatives bearing four methoxy substituents showed that when all four substituents were in *ortho* positions relative to the azo unit, and there was an amino group in the para position (e.g. **6**), the thermal half-life of the *cis* isomer was appropriate (~20 s) but the wavelength maximum was too short so that absorbance at wavelengths >700 nm was minimal.[17] An *ortho-meta* arrangement of the methoxy groups (**7**), in contrast, produced a substantial red-shift, but led to a very short (μs) *cis* isomer thermal half-life and a pK_a for the azonium ion too low for use at pH 7.0.[18]

The neutral *trans* form of tetra-*ortho* substituted azo compounds such as **6**, exhibits a highly twisted geometry,[17,19] a feature that is absent in the *ortho-meta* derivative.[18] The steric hindrance that produces this twisting may also increase the thermal energy barrier for *cis*-to-*trans* isomerization. Thus, to obtain slow thermal relaxation together with absorbance in the bio-optical window, both tetra-*ortho* substitution and *meta*-methoxy substitution would appear necessary, for example as in **8**, which bears methoxy substituents at all *ortho* and all *meta* positions. Computational modeling showed that steric clash of the methyl groups in **8** would lead to loss of conjugation between methoxy oxygen lone pairs and the ring systems. Locking the conformation of the methoxy groups by creating dioxane rings (compound **9**) solves this problem.

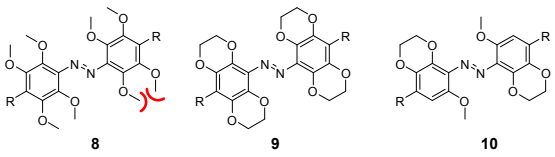


Chart 1.

Computational analysis indicated that *para* amino substituted derivatives of **9** would have the desired red shift (see SI). However, precursors to **9** had extremely poor solubility in a variety of solvents so that further derivatization proved impractical. The substitution pattern shown in compound **10**, in which there is one *meta* oxygen substituent per ring, was then considered. Computational modeling indicated that *para* amino substituted derivatives of **10** would exhibit the twisted *trans* neutral state, as well as a planar *trans* azonium ion expected for tetra-*ortho* substituted methoxy azobenzenes (Fig. 1). Due to the asymmetry of the compound, distinct conformations exist with the dioxane rings on the same side or on different sides of the molecule. For the *trans* azonium form of the compound, these conformations were calculated to differ by less than 1 kcal/mol in energy (see SI); the lowest energy conformation is shown in Figure 1b with a stabilizing H-bond indicated. TD-DFT calculations gave a predicted absorbance maximum for this species near 600 nm, *i.e.* 50 nm further red-shifted than **6**, so that substantial absorbance >700nm was expected (see SI).

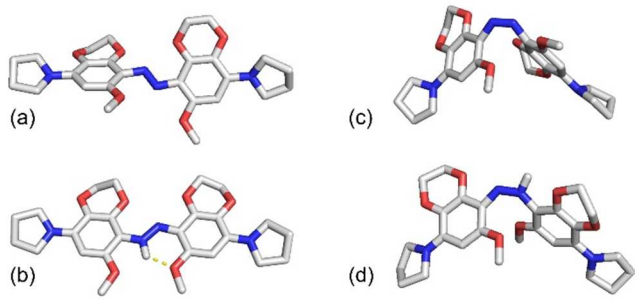
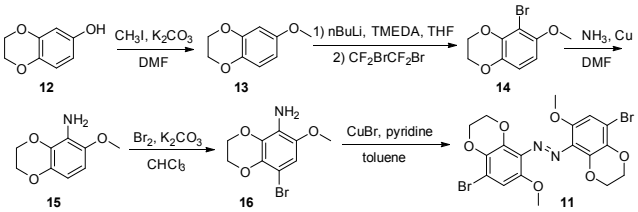


Figure 1. DFT-calculated low energy conformations of a *p*-pyrrolidine derivative of **10** in the (a) neutral trans form (b) *trans* azonium form, (c) neutral *cis* form, (d) *cis* azonium form.

Synthesis

The synthetic route to the *para*-Br derivative of **10**, (designated **11**), which serves as a precursor for *para*-amino derivatives is shown in Scheme 1. This employs a key *ortho*-lithiation step to convert **13** to **14**. A similar approach has been exploited recently by Feringa to develop general approaches for the synthesis of *ortho* substituted azobenzenes.[2] The structure of the final compound was confirmed by X-ray crystallography (Fig. 2).



Scheme 1. Synthesis of the dioxane-methoxy-azobenzene (DOM-azo) core.

As had been observed with the related *tetra-ortho* methoxy substituted compound,[19] compound **11** can co-crystallize with chloroform with the acidic proton of the $CHCl_3$ molecule making H-bond interactions with the methoxy oxygen atoms and the azo nitrogen atoms (Fig. 2a). Spectra of *trans* and *cis* isomers of the non-

protonated form of **11** in organic solvents (Fig. 2b) show the separation of n-π* transitions expected for a tetra-*ortho* substituted azobenzene derivative (see SI).[19,20]

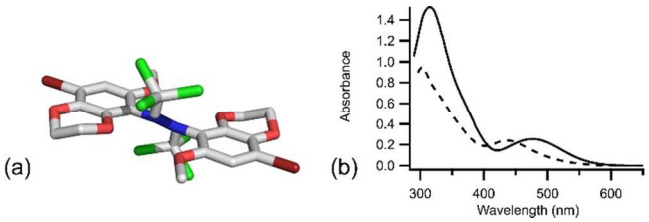


Figure 2. (a) X-ray structure of **11**, co-crystallized with $CHCl_3$ (b) UV-Vis spectra of dark-adapted (solid line) and irradiated (540 nm, dashed line) in toluene.

Since the nature of the amine in the *para* position can tune the wavelength maximum and also the azonium pK_a, a variety of derivatives of **10** were then prepared using Buchwald-Hartwig coupling to link various amines to the *para*-Br compound **11** (see SI). The structures of these are shown in Table 1. Polar groups were included to promote water solubility.

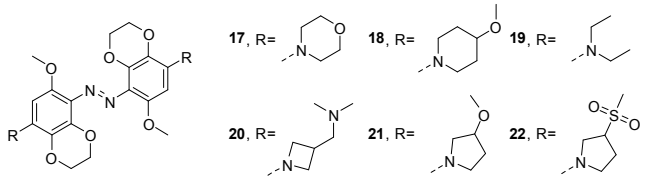


Table 1. Properties of *trans* azonium ions formed by *p*-amino substituted derivatives of **10**

Structure	λ_{max}	pK_a
17	569	4.5
18	560	5.5, 3.5*
19	580	7.1, 6.2*
20	595	6.4
21	575	~7
22	597	6.7

*Second pK_a due to formation of ammonium species.

Spectra of *trans* isomers in aqueous solutions and pK_as

Acid-base (pH) titrations in aqueous solutions were carried out for the thermally stable *trans* isomeric forms of the compounds shown in Table 1 (see SI for details). The pK_a of **17** was found to be well below the physiological range. Compound **18** gave a higher pK_a, consistent with better donating ability of the *p*-amino substituent, but this was still below 7 and below the corresponding tetra-*ortho* methoxy compound (*i.e.* the compound without a substituent in the *meta* position).[18] The low pK_a is thus likely due to steric clash between H-atoms on the 6-membered *p*-amino substituent and the benzodioxane ring leading to twisting of the 6-membered N-containing ring relative to the aromatic ring and diminished delocalization of the nitrogen lone pair into the azo system. Twisting of the 6-membered N-containing ring relative to the aromatic ring is confirmed by computational modeling.[18] The diethylamino-substituted derivative **19** exhibited a substantially higher pK_a (7.1), but had an unexpectedly elevated second pK_a for the ammonium species (6.2). This species absorbs at shorter wavelengths (see SI) and the coexistence of azonium and ammonium species at physiological pH means that absorbance at far-red wavelengths is diminished. The azetidine-substituted compound **20** and the pyrrolidinesubstituted compounds **21**, and **22** exhibited pK_a values close to neutral and strongly red-shifted maximal absorbance. Compound **21** was prone to aggregation, however, especially in pH ranges near the pK_a. This observation suggests that aggregation may involve sharing of protons between molecules. Of course, the solubility properties are likely to be different when the photoswitch is part of a larger bioactive molecule.

Compound **22**, in which the *para* substituent is a pyrrolidine group bearing a solubilizing sulfone moiety, proved much less prone to self-association. The compound also exhibited the longest wavelength absorbance tail, together with a pK_a near neutral. Figure 3 shows the acid-base titration of this compound, which was designated DOM-azo (for dioxane-methoxy-azobenzene). The observed pK_a of 6.7 (Fig. 3b) means that ~25% of the molecules are protonated at a physiological pH of 7.2. The molar absorptivity of the azonium ion is high (138,000 $M^{-1}cm^{-1}$ at 600 nm)(see SI) so that strong absorbance is seen at wavelengths >700 nm at physiological pH.

We then tested the ability of DOM-azo to photoswitch upon exposure to near-IR light. A high intensity LED (17 mW/cm^2) with a maximum emission at 720 nm was used to irradiate a solution of DOM-azo at pH. 7.2, and a 540 nm LED source was used as a measuring beam (the spectra of these LED sources is shown in Fig. 3a for reference). A photomultiplier tube was used to monitor changes in absorbance over time. Figure 3c shows photoswitching time courses in response to 720 nm irradiation interleaved with periods of thermal relaxation in the dark. Clear, rapid photoswitching is seen (τ_{on} = 0.9 ± 0.1 s) together with thermal relaxation (τ_{off} 0.7 ± 0.1 s). These time courses are rapid enough to permit spatial localization of the *cis* isomer *in vivo*.

Finally, we tested the stability of DOM-azo in water by measuring absorbance scans over extended periods of time and found no significant changes over >6 h (see SI). Thin layer chromatography of solutions of DOM-azo stored in aqueous buffer at physiological pHs showed the compound was intact after 3 months at room temperature (see SI). We then tested stability to glutathione, the primary intracellular reducing agent. A slow loss of colour ($\tau_{1/2}$ ~3 h) was observed when DOM-azo was exposed to 10 mM reduced glutathione (see SI), the highest concentration expected intracellularly.[21] This sensitivity to reduction is similar to that observed for related azonium ions previously[17] and may limit these photoswitches to extracellular applications, for example as blood borne photopharmaceuticals interacting with extracellular receptors.

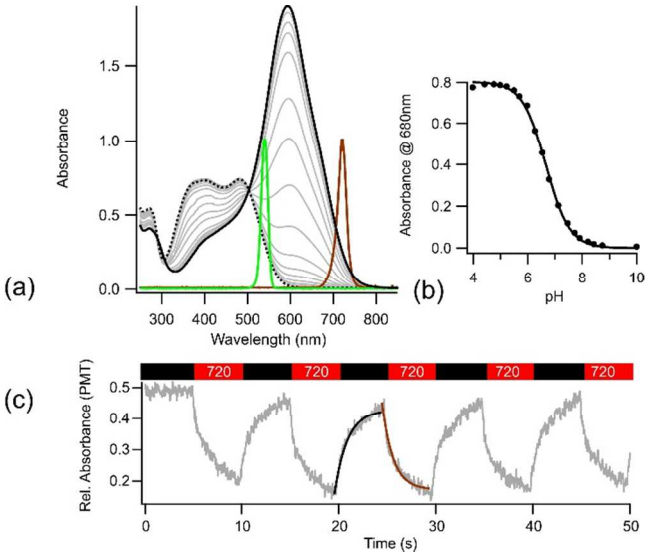


Figure 3. (a) UV-Vis spectra of DOM-azo (**22**) as a function of pH. The solid black line is the limiting spectrum of the azonium ion at low pH (<pH 5.0); the dotted black line is the spectrum of the neutral *trans* isomer obtained at pH 10.0. Spectra at intermediate pHs are shown in gray. (b) Absorbance (680 nm) plotted vs. pH from the data in part (a); the fitted pK_a = 6.7. (c) Photoswitching with near-IR light under physiological conditions (aqueous buffer solution (20% DMSO), pH 7.2). Absorbance at 540 nm was monitored vs. time while the sample was exposed to 720 nm near-IR light (indicated by red bars above the trace) or left in darkness (black bars). On and off time constants were extracted from exponential fits (two such fits are indicated). The spectral outputs of the near-IR LED used for switching (red line) and the green LED used to monitor absorbance (green line) are shown (red line) in part (a).

CONCLUSION

By tailoring the structure of an azobenzene with appropriate substituents, *trans*-to-*cis* photoswitching was achieved with near-IR light (720 nm) from a continuous LED source under physiological conditions. The core photoswitch structure developed here may form a basis for future photopharmaceuticals. It could directly enable near-IR responses in current photoswitchable tethered ligands for ion channels and receptors,[22] in photoswitchable cross-linkers[23] or in azobenzene -based drug delivery systems.[24]

ASSOCIATED CONTENT

Supporting Information. Details of computational modeling of structure and absorbance properties, details of syntheses, acid base titrations, and photoswitching experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

DOM, dioxane-methoxy; IR, infrared; LED, light emitting diode; MRI, magnetic resonance imaging, PET, positron emission tomography; TLC, thin layer chromatography; UV, ultraviolet.

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SYNOPSIS TOC

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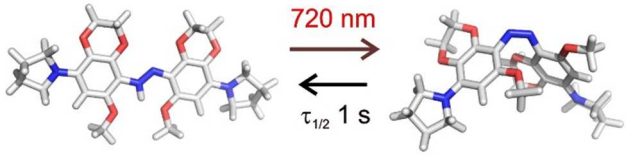

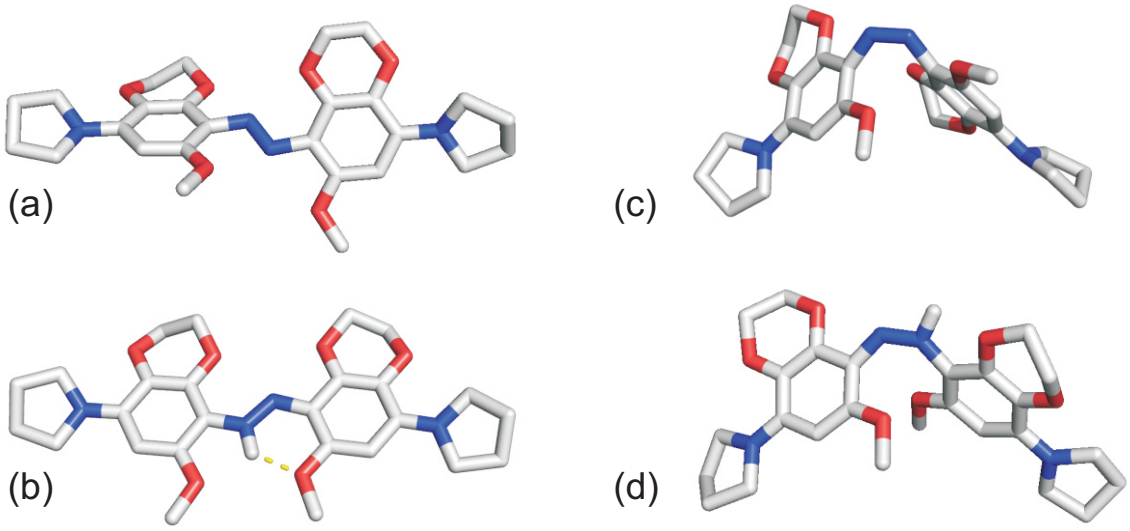
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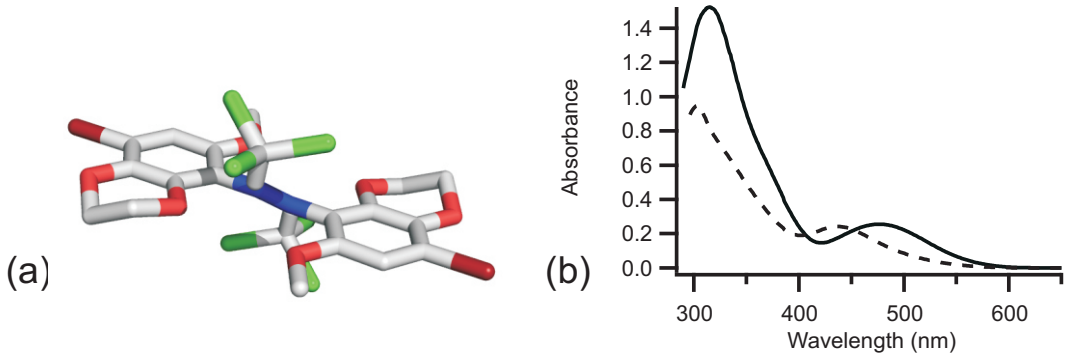
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(b)
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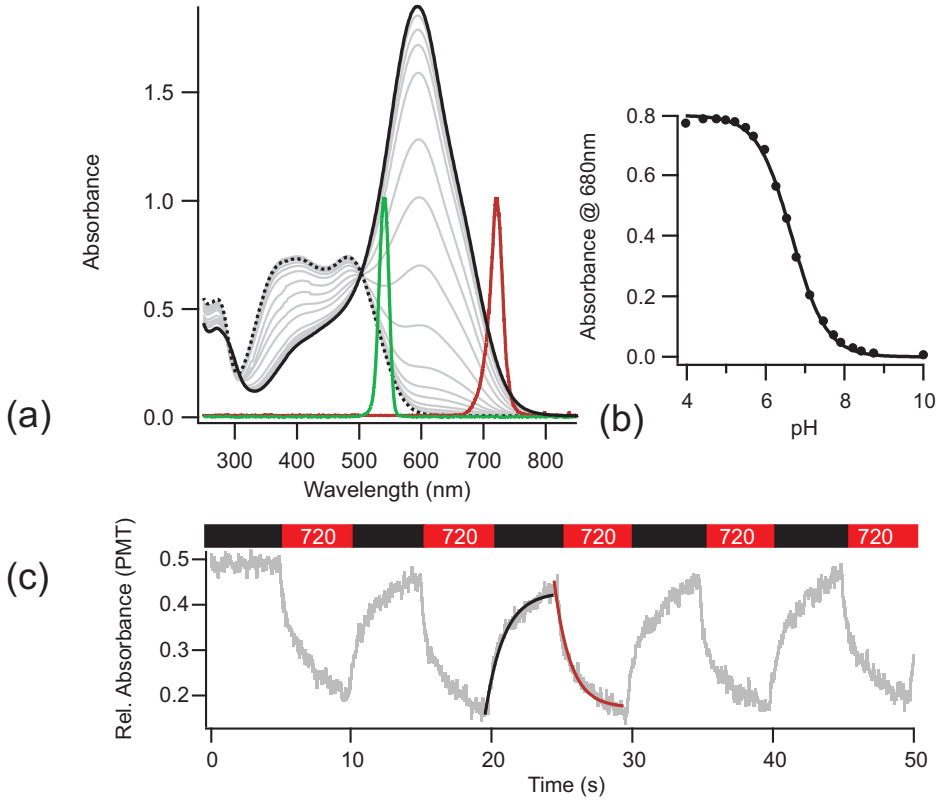
Wavelength (nm)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300
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500
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Wavelength (nm)
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0.6
0.4
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8
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pH
(c)
720
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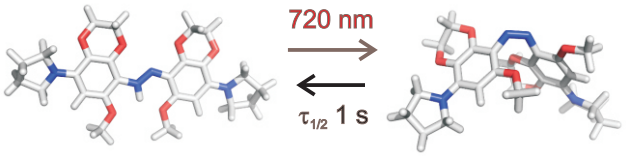
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