Photoisomerization

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Bidirectional Photocontrol of Peptide Conformation with a Bridged Azobenzene Derivative**

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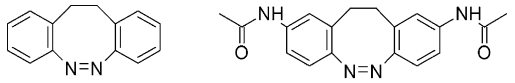
A major challenge in photocontrol is to combine a large conformational change with complete bidirectional photoswitching.[1] The azobenzene chromophore undergoes a large conformational change upon isomerization but, because of the overlap of the absorbance spectra of the *cis* and *trans* isomers, photoswitching is incomplete.[2] Thermal isomerization can fully repopulate the thermodynamically more stable *trans* state, but this process may be too slow for many applications. Increasing the thermal relaxation rate by introducing appropriate ring substituents is possible,[3] but means that one cannot maintain the *cis* isomer without continual irradiation.

In 2009 Siewertsen et al. reported the remarkable photochemistry of the bridged azobenzene derivative **1**.[4] This compound had been described 100 years earlier, but its photochemical properties had apparently not been realized.[5] The C_2 bridge in **1** produces a highly twisted *trans* isomer that is less stable than the *cis* isomer—opposite to the situation with normal azobenzenes. Most importantly, from the point of

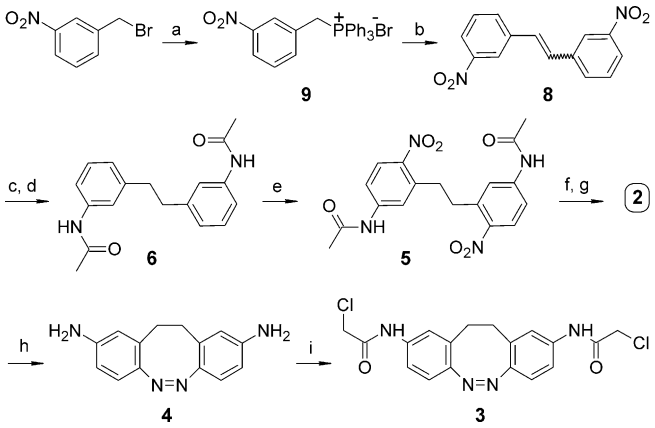


1 **2**

view of photocontrol, the C_2 bridge leads to an 85 nm separation of the n-π* absorption bands of the *cis* and *trans* isomers, so that there are wavelengths where only the *trans* isomer absorbs. The separation of the n-π* transitions of *cis* and *trans* isomers has also been seen recently with tetra-*ortho*-methoxy-substituted azobenzene derivatives,[6] although in this case the separation is only 35 nm.

The large separation of the n-π* transitions of C_2-bridged azobenzene **1** would be even more useful from the standpoint of biomolecular photoswitching if the *cis*-to-*trans* switching wavelength (where $\varepsilon_{cis}/\varepsilon_{trans}$ is maximal) were slightly red-shifted so that it was in the visible range rather than in the UV region.[4] An additional concern for biological studies is the stability of the azo derivative to reduction by glutathione, which is present intracellularly at concentrations between 1 and 10 mM.[7] We reasoned, based on our experience with the substitution of azobenzene itself,[8] that *p*-acetamido substitution (i.e. compound **2**) might result in a suitable red-shift and confer stability against reduction by glutathione.[9] Moreover, *p*-acetamido substitution permits the synthesis of a thiol-reactive cross-linker version (**3**) of the photoswitch that is suitable for introduction into biomolecules such as peptides and proteins.[10]

Scheme 1 shows the synthetic approach we used to produce the *p*-acetamido-substituted C_2-bridged azobenzene **2** and the thiol-reactive chloroacetamido derivative **3**. A



Scheme 1. Synthesis of bridged azobenzene photoswitches **2** and **3**. a) PPh_3, toluene, reflux, yield 96%; b) *t*BuOK, 3-nitrobenzaldehyde THF, 0°C–RT, 57%; c) H_2, Pd/C, MeOH, yield 90%; d) Ac_2O, Py, THF, yield 78%; e) KNO_3, $AcOH/H_2SO_4$ (conc.), 0°C–RT, 31%; f) $Ba(OH)_2{\cdot}8H_2O$, Zn, EtOH, reflux; g) HgO, EtOH, reflux (overall yield for two steps 8%); h) KOH, MeOH, reflux, 70%; i) chloroacetic anhydride, Py, diethyl ether, 50%. Py = pyridine.

Wittig reaction was used to produce 1,2-bis(3-nitrophenyl)-ethene (**8**) from (3-nitrobenzyl)triphenylphosphonium bromide (**9**) and 3-nitrobenzaldehyde. The alkene and the two nitro groups of **8** were reduced using H_2-Pd/C to give 1,2-bis(3-aminophenyl)ethane (**7**; see the Supporting Information). This compound was acetylated (to give **6**) and then nitrated to give 1,2-bis(2-nitro-5-acetamidophenyl)ethane (**5**). A two-step oxidation protocol, adapted from that of Paudler and Zeiler,[11] using $Ba(OH)_2$, Zn, and HgO led to *para*-diacetamido-substituted C_2-bridged azobenzene **2**. Hydrolysis of the two acetamido groups of **2** with KOH resulted in *para*-diamino-substituted C_2-bridged azobenzene **4**, which was treated with chloroacetic anhydride/pyridine to give *para*-di(α-chloroacetamido)-substituted C_2-bridged azobenzene **3**.

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Computational (B3LYP/cc-pVTZ) methods were used to calculate the relative stability of the *cis* and *trans* isomers of **2**, and these calculations confirmed that, similar to the parent unsubstituted C_2-bridged compound **1**, the *cis* isomer was the more stable by approximately 8 kcal mol^{-1} (see the Supporting Information). An X-ray crystal structure of the *cis* isomer confirmed the calculated structure (Figure 1). Time-dependent DFT calculations also supported our expectation that the *p*-acetamido substituent would red-shift the wavelengths of both the *cis* and *trans* n-π* transitions (see the Supporting Information). Figure 1 shows the UV/Vis spectra of *cis*-**2**, prepared in the dark, in DMSO where the n-π* transition is approximately 10 nm red-shifted compared to that of the parent bridged compound.[4] Irradiation of this species produced the *trans* isomer, which exhibited an n-π* transition maximum near 505 nm (Figure 1), approximately 15 nm red-shifted compared to the parent compound and 90 nm red-shifted compared to the *cis* form.

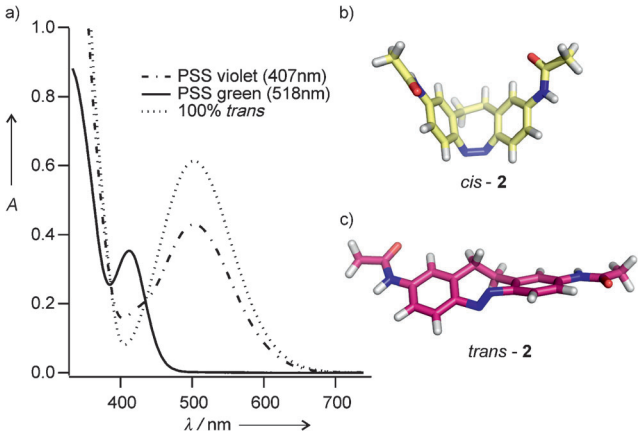

Figure 1. a) UV/Vis spectra of the photostationary states (PSS) obtained for **2** in DMSO. b) X-ray crystal structure of *cis*-**2**. c) Calculated (B3LYP/cc-pVTZ) structure of *trans*-**2**.

The *trans* isomer was most effectively produced by irradiation at 407 nm, the wavelength where $\varepsilon_{cis}/\varepsilon_{trans}$ was maximal. This wavelength is conveniently available in many low-cost violet light-emitting diodes (LEDs) and microscope illuminators, and has been chosen in other studies as a target wavelength for the design of photoswitches.[12] Irradiation at 407 nm produced a photostationary state that was 70:30 *trans*/*cis* as judged by NMR spectroscopy (see the Supporting Information). The extrapolated spectrum of the pure *trans* isomer is also shown in Figure 1. Thermal relaxation from the *trans* to the *cis* isomer occurred with a half-life of about 4.8 h at 20 °C in DMSO (see the Supporting Information), a rate similar to that of the parent bridged compound.[4] Importantly, the spectrum of the *trans* isomer has a long wavelength tail where the *cis* isomer essentially does not absorb (see the Supporting Information for the absorbance spectrum of a very concentrated solution of the *cis* isomer at long wavelengths). This feature means that irradiation with green light (500–550 nm) completely (> 99.7 %) repopulates the more stable *cis* isomeric state. Thus, a photocontrolled change in the concentration of the *trans* isomer of at least 400-fold (see the Supporting Information) is possible. The large separation of the n-π* bands of the *cis* and *trans* isomers also results in a convenient visible color change of these solutions from pale yellow for the *cis* to reddish purple for the *trans* isomer.

The photoisomerization of azobenzene has been used to control a wide variety of biomolecular targets (peptides, proteins, and nucleic acids) in vitro[10,13] as well as in cell extracts.[14] It has been applied to the photocontrol of proteins in living cells and tissues[15] and to the photocontrol of ion channels in vivo.[16] Photocontrol of the conformation of helical peptides has been extensively investigated as a prototype for conformational control, where the mechanism of the coupling of the azo isomerization to the peptide conformational response can be well described.[17,18] Peptides bearing pairs of Cys residues can be intramolecularly cross-linked with thiol-reactive azobenzene-based photoswitches such as **3** (Figure 2). When a peptide bearing a pair of Cys residues undergoes a helix–coil transition, the spacing between the Cys thiol groups changes. The greatest degree of photocontrol over the peptide conformational can be achieved when the spacing between the Cys thiol residues in the helical state of the peptide matches one isomer of the photoswitch but not the other.[10,18]

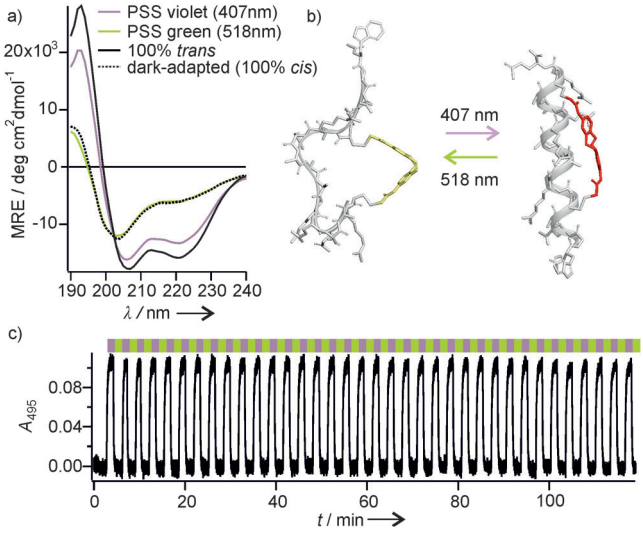

Figure 2. Photoswitching of helical peptide conformation with the bridged azobenzene derivative **3**. a) CD spectra obtained with irradiation conditions shown at 20 °C (5 mM Na phosphate buffer, pH 7.0). b) Models showing FK-11 (AcWGEACAREAAAREAACRQ-NH_2) cross-linked with **3** in the *cis* (left) and *trans* (right) conformations. c) Multiple rounds of photoswitching (monitored by the absorbance of the *trans* isomer) can be carried out in the presence of 5 mM reduced glutathione.

To investigate optimal Cys spacings for the bridged photoswitchable cross-linker **3** we performed molecular dynamics simulations on the isolated cross-linker (see the Supporting Information). These simulations indicated that the mean end-to-end distances are somewhat shorter for both isomers compared to their nonbridged counterparts,[19] but

the change in the end-to-end distance is similar for the bridged and nonbridged photoswitches. The simulations indicated that the *trans* isomer has an end-to-end distance that matches well with the thiol (S-S) spacing in a helical peptide with Cys residues spaced i, i + 11 in the primary sequence. This distance is too long to be spanned by the *cis* isomer of **3**. In the case of the bridged photoswitch, the *cis* isomer is the thermodynamically more stable state, thus the nonhelical conformation is expected to be preferred—opposite to the case for the nonbridged analogue.[20]

We synthesized the peptide FK-11, known to be a helix-forming peptide in aqueous solution,[20] and cross-linked it with **3**. Figure 2 shows the circular dichroism (CD) spectra for the cross-linked peptide after irradiation at 407 nm and 518 nm. These spectra indicate that, as expected, irradiation with violet light causes an increase in the helix content for FK-11 cross-linked with **3**, while irradiation with green light completely reverses this conformational change (Figure 2). The thermal relaxation from the *trans* to the *cis* isomer was slower when the photoswitch was attached to the peptide ($\tau_{1/2}$ = 8.3 h at 20 °C, compared to 4.8 h for **2** in DMSO; see the Supporting Information). A CD signal is also seen at longer wavelengths for the *trans* isomer (not for the *cis* isomer; see the Supporting Information). This observation of induced CD indicates that the n-π^* transition in the *trans* isomer senses the chiral environment of the peptide.

As with the parent bridged azobenzene derivative studied by Siewertsen et al., the *p*-acetamido derivative was found to be photostable, undergoing hundreds of cycles of irradiation with violet and green light without noticeable decay, both as a free compound in DMSO and when attached to the peptide in aqueous solution (see the Supporting Information). We then tested the redox stability of the *p*-acetamido-substituted bridged azobenzene when attached to the FK-11 peptide by exposing it to various concentrations of reduced glutathione under different irradiation conditions. The *cis* isomer was found to be completely stable to overnight incubation with 10 mM reduced glutathione (see the Supporting Information). The *trans* isomer, however, underwent a bleaching process, whose rate depended on the concentration of the reduced glutathione (see the Supporting Information). The process was slow enough, however, under typical physiological conditions ($\tau_{1/2} \approx 3$ h at 25 °C, 5 mM GSH, pH 7.0) that multiple cycles of photoswitching could be carried out without a measurable decay of the signal (Figure 2). This sensitivity of the *trans* isomer to reduction is likely a result of its highly strained nature relative to the nonbridged analogue, which is not reduced under comparable conditions.[9]

These results demonstrate that the bridged azobenzene derivative **3** is an effective photoswitch for the photocontrol of peptide conformation with visible light. This photoswitch has the particular advantage that the large separation of the absorbance bands of the *cis* and *trans* isomers enables complete bidirectional photoswitching—violet light produces 70 % *trans* and green light produces > 99.7 % *cis*. The slow thermal isomerization process of the peptide-bound photoswitch in aqueous solution, together with the excellent resistance of the *cis* isomer to reduction by glutathione means that bioactive peptides could be held in an "off" (e.g. nonhelical) conformational state for long periods of time, then pulsed "on" (helical) with violet light and "off" again with green light. Such photocontrolled biomolecules are expected to find applications in diverse settings.[21]

Experimental Section

The peptide FK-11 (AcWGEACAREAAAREAACRQ-amide) was prepared by using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid-phase synthetic methods. Intramolecular cross-linking of cysteine residues in FK-11 with **3** (prepared as described in the Supporting Information) was performed in 25 % DMSO as follows: A solution of 0.5 mM peptide (freshly purified) and 2 mM cross-linker **3** in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8 was stirred at 37 °C under a nitrogen atmosphere for 24 h. The completion of the reaction was judged by ESI mass spectrometry. The reaction was dried under high vacuum, and the cross-linked peptide was purified by reverse-phase HPLC. FK11 cross-linked with **3** was eluted at 46 % acetonitrile/water and characterized by ESI-MS: *m*/*z* calcd for $C_{98}H_{142}N_{34}O_{28}S_2$: 2308.53 [$M^+$]; found: 2308.71. Details of the UV/Vis, CD, and NMR spectroscopy measurements are provided in the Supporting Information.

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