Azobenzene Photoswitching without Ultraviolet Light.

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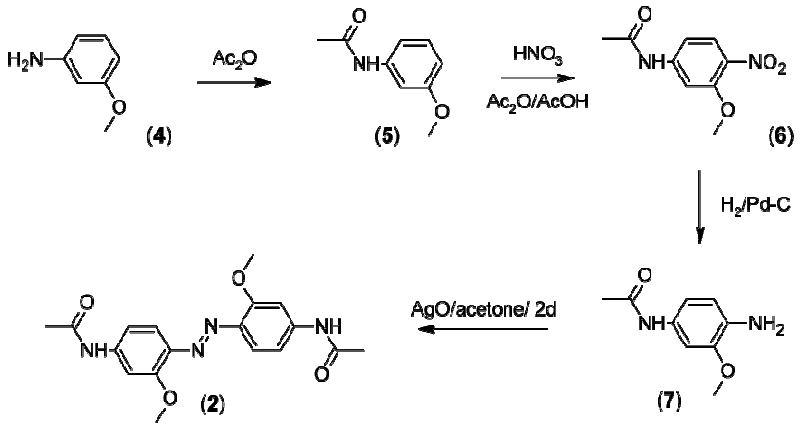
Supporting Information

Synthesis: The parent compound (**1**) was synthesized as described previously.[1] The dimethoxy derivative (**2**) was synthesized as outlined in the scheme below:

i) Synthesis of 2,2'-dimethoxy-4,4'diamidoazobenzene (2)



***N*-(3-methoxyphenyl)acetamide** (**5**) was prepared according to Akhavan-Tafti, H. et al.[2] Acetic anhydride (20 mL; 212.0 mmol) was added to a mixture of 3-methoxyaniline (**4**) (*m*-anisidine) (Aldrich) (20 g, 162.4 mmol) in 20 mL of acetic acid at 0^0C. The reaction was stirred overnight at room temperature then poured into 100 g of ice in 100 mL of water. The resulting solid was collected by filtration (Yield: 93%) and used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.13 (s, 3H), 3.76 (s, 3H), 6.63 (dd, J= 8.0, 1.8 Hz, 1H), 6.95 (dd, J= 8.0, 1.0 Hz, 1H), 7.17 (t, J= 8.0 Hz, 1H), 7.25 (m, 1H), 7.48 (br s, 1H).

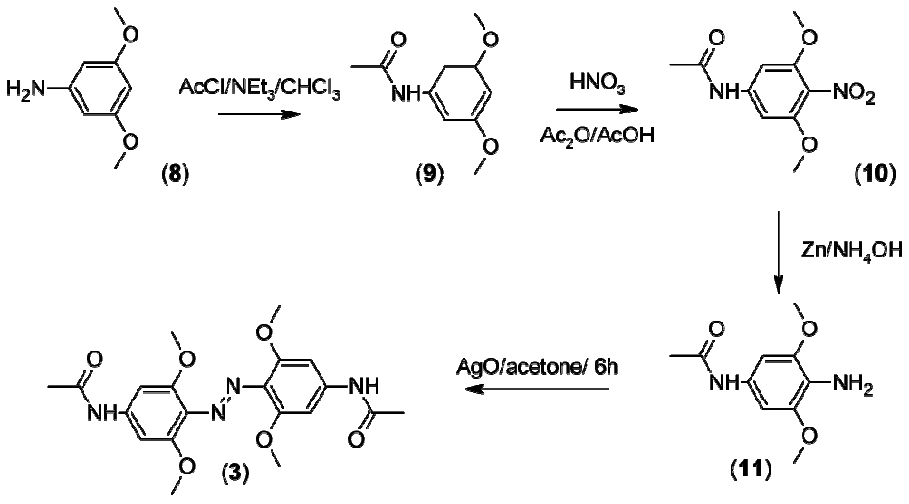
***N*-(3-methoxy-4-nitrophenyl)acetamide (6)**. To 1 g (6 mmol) of *N*-(3-methoxyphenyl)acetamide (**5**) in 30 ml of acetic anhydride, a solution of $HN0_3$ (3 mL, d = 1.42) in acetic acid was added dropwise over a 3 h period at 0-5 ^{0}C. Once TLC indicated the reaction was complete, the reaction mixture was poured into 100 g of ice in 100 mL of water. The resulting solid was collected by filtration, then dried and purified by chromatography on silica with a $CHCl_3$-MeOH mixture. (Yield: 20%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.10 (s, 3 H) 3.87 (s, 3 H) 7.24 (dd, *J*=9.0, 2.1 Hz, 1 H) 7.65 (d, *J*=2.1 Hz, 1 H) 7.93 (d, *J*=9.0 Hz, 1 H) 10.45

(s, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 24.96, 56.97, 103.66, 110.73, 127.69, 133.86, 146.11, 154.65, 170.04. HRMS-ESI calc'd (MH+) ($C_9H_{10}N_2O_4$) 211.0713 Da; obs'd 211.0744 Da

***N*-(4-amino-3-methoxyphenyl)acetamide (7)**. A solution *N*-(3-methoxy-4-nitrophenyl)acetamide (**6**) (1 g, 4.7 mmol) in ethyl acetate (30 ml) was treated with 5% palladium on charcoal. The mixture was hydrogenated at room temperature and pressure for 3 days. The catalyst was removed by filtration and the filtrate was evaporated to give the title compound as a pale green solid. (Yield: 90%). An analytical sample was purified by column chromatography on silica with chloroform/methanol 30/1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.96 (s, 3 H) 3.72 (s, 3 H) 4.45 (s, 2 H) 6.53 (d, *J*=8.4 Hz, 1 H) 6.84 (dd, *J*=8.3, 2.2 Hz, 1 H) 7.13 (d, *J*=2.2 Hz, 1 H) 9.53 (s, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 24.46, 55.84, 104.30, 112.77, 114.10, 129.99, 134.03, 146.61, 167.92. HRMS-ESI calc'd (MH+) ($C_9H_{12}N_2O_2$) 181.0971 Da; obs'd 181.1000 Da

***N*,*N*'-[(*E*)-diazene-1,2-diylbis(3-methoxybenzene-4,1-diyl)]diacetamide (2)**. Freshly prepared, dry AgO (1.04 g, 8.4 mmol)[3] was added to a solution of N-(4-amino-3-methoxyphenyl)acetamide (**7**) (0.7 g, 3.8 mmol) in dry acetone (25 mL) at room temperature with vigorous stirring. After stirring in the dark for 1d, a fresh portion of AgO (1.04 g) was added, stirring was continued for another 1d, and the presence of the initial amine was monitored by thin-layer chromatography. The solid was collected by filtration, washed with methanol, and the filtrates were combined and evaporated. The product was purified by column chromatography on silica gel ($CHCl_3$/MeOH 20/1; Rf~0.3) to give 0.11 g (Yield: 16%) of a fine orange solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.08 (s, 6 H) 3.90 (s, 6 H) 7.16 (dd, *J*=8.8, 1.7 Hz, 2 H) 7.47 (d, *J*=8.9 Hz, 2 H) 7.64 (d, *J*=1.6 Hz, 2 H) 10.23 (s, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 24.65, 56.25, 103.50, 111.29, 117.27, 138.11, 143.54, 157.51, 169.19. HRMS-ESI calc'd (MH+) ($C_{18}H_{20}N_4O_4$) 357.1557 Da; obs'd 357.1577 Da.

ii) Synthesis of 2,2',6,6'-tetramethozy-4,4'diamidoazobenzene (3)



***N*-(3,5-dimethoxyphenyl)acetamide (9).** To a stirred solution of 25g (0.16 mol) of 3,5-dimethoxyaniline (**8**) (Aldrich) dissolved in 400 ml of dry tetrahydrofuran containing 68 ml (0.49 mol) of triethylamine, 12.8 ml (0.17 mol) of acetylchloride was slowly added at 5^{o}C. The mixture was stirred at room temperature for 16 hours. The triethylamine hydrochloride was filtered off, while the organic phase was concentrated and the product was separated by column chromatography (silica gel, CH_2Cl_2/ EtOAc (10:1)), (Yield: 94%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.97 (s, 3 H) 3.66 (s, 6 H) 6.15 (t, *J*=2.34 Hz, 1 H) 6.79 (d, *J*=2.34 Hz, 2 H) 9.83 (s, 1 H).

***N*-(3,5-dimethoxy-4-nitrophenyl)acetamide (10)** A solution of 70% nitric acid (1 ml) in 2 ml of acetic acid was added dropwise to a solution of *N*-(3,5-dimethoxyphenyl)acetamide (**9**)(3 g, 0.0153 mol) in 200 ml of acetic anhydride at 0 $^{\circ}$C. The reaction was stirred for 1hour at a temperature not higher than 7 $^{\circ}$C, and then 3 hours at room temperature. The mixture was then evaporated at room temperature to 10-15% of the initial volume. The solution was cooled, mixed with water (200ml) and extracted with EtOAc (3x100). The combined extract was dried (anhydrous Na_2SO_4) and evaporated, with the resulting residue then purified by chromatography on silica gel, $CHCl_3$/MeOH (30:1). Yield: 12%. ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 2.14 (s, 3 H) 3.84 (s, 6 H) 7.07 (s, 2 H). ^{13}C 13C NMR (100 MHz, methanol -d_4) δ ppm 22.97, 55.75, 95.52, 116.63, 141.96, 152.29, 170.77. HRMS-ESI calc'd (MH+)($C_{10}H_{12}N_2O_5$) 241.0819 Da; obs'd 241.0815 Da.

***N*-(4-amino-3,5-dimethoxyphenyl)acetamide (11)** 1.3 g of *N*-(3,5-dimethoxy-4-nitrophenyl)acetamide (**10**) was mixed with 150 ml of ammonium hydroxide (30%). To this mixture 5 g of Zn dust was added with 1-2 min of vigorous stirring. The mixture was left to stir at 35 $^{\circ}$C for 20-40 min. After that time the mixture was filtered and the liquid was evaporated until half of the initial volume remained. Filtration was repeated and the liquid was then extracted with $CHCl_3$ (3x150ml). The organic phase was dried with Na_2SO_4 and evaporated. The product contained some unprotected aniline. Further purification was done with silica column. $CHCl_3$/MeOH (2:1) Yield: 71% (0.812g). ^{1}H NMR (400 MHz, chloroform-*d*) δ ppm 2.12 (s, 3 H) 3.80 (s, 6 H) 6.73 (s, 2 H) 7.40 (br. s., 1 H). ^{13}C NMR (100 MHz, chloroform-*d*) δ ppm 24.60, 56.06, 97.99, 122.39, 128.84, 147.42, 168.53. HRMS-ESI calc'd (MH+) ($C_{10}H_{14}N_2O_3$) 211.1077 Da; obs'd 211.1076 Da.

N-{4-[(E)-2-(4-acetamido-2,6-dimethoxyphenyl)diazen-1-yl]-3,5-dimethoxyphenyl}acetamide (3) 0.8g of *N*-(4-amino-3,5-dimethoxyphenyl)acetamide (**11**) was dissolved in 80 ml of dry acetone and to this 1.3g of AgO was added. The mixture was stirred overnight at room temperature in dark. If the reaction was not complete after 24 hours then an additional 1g of AgO was added and stirred for the next 24 hours. After this time the mixture was filtered and the solid was washed with acetone and MeOH (do not use with chloroform, since this results in a fire of the filter within 5-10 min after washing!). The liquid was evaporated, dissolved in chloroform and purified on a silica column with $CHCl_3$/ MeOH (100:6). Yield: 0.086g (10.8%). $CHCl_3$/MeOH (2:1). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.99 (s, 6 H)(cis), 2.07 (s, 6 H)(trans), 3.49 (s, 12 H)(cis), 3.68 (s, 12 H)(trans), 6.84 (s, 4 H)(cis), 7.09 (s, 4 H)(trans), 9.91 (s, 2 H)(cis), 10.12 (s, 2 H)(trans). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 24.08 (cis), 24.20 (trans), 54.91 (cis), 55.91 (trans), 94.82 (cis), 95.90 (trans), 129.42 (trans), 129.70 (cis), 139.34 (cis), 140.62 (trans), 149.31

(cis), 152.20 (trans), 168.32 (cis), 168.62 (trans). HRMS-ESI calc'd (MH+) ($C_{20}H_{24}N_4O_6$) 417.1768 Da; obs'd 417.1757 Da.

Spectra and Photoisomerization:

i) Measurement of UV/Vis spectra and thermal relaxation rates

Ultraviolet absorbance spectra were obtained using either a Perkin-Elmer Lambda 35 spectrophotometer or a diode array UV–Vis spectrophotometer (Ocean Optics Inc., USB4000) coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc.). Measurements of thermal relaxation rates used the former arrangement, while steady state spectra used the latter. Spectra were acquired in a 1 cm pathlength quartz cuvette in a temperature-controlled cuvette holder and the sample was irradiated (at 90° to the measuring beam) with LEDs emitting at 370 nm or 460 nm or 530 nm (370 nm, 897-LZ440U610 LedEngin LED; 530 nm, Luxeon LXHL-LM5C; royal blue (460 nm, Luxeon LXHL-LR5C) until no further decrease in absorbance was observed. Irradiation was also carried out using a 103W/2 short arc mercury lamp (3000 lumens) with a Cy3 filter set (ex. 530-560 nm).

Rates of thermal cis-to-trans isomerization at different temperatures were measured by acquiring scans as a function of time after irradiation to convert a percentage of the solution to the cis isomer. Rates were measured at high temperatures (50-70°C) and those reported were then obtained by extrapolation of an Arrhenius plot.

Dark-adapted spectra of (**3**) were obtained by equilibrating the sample in the dark in 50 mM ammonium acetate buffer pH 3.0 for 30 minutes at 40°C. Under these conditions the thermal cis to trans rate increases substantially ($\tau_{1/2}$ ~6 min). The sample was then lyophilized and dissolved in 25 mM sodium phosphate buffer pH 7.0 or DMSO for UV/Vis spectra measurements.

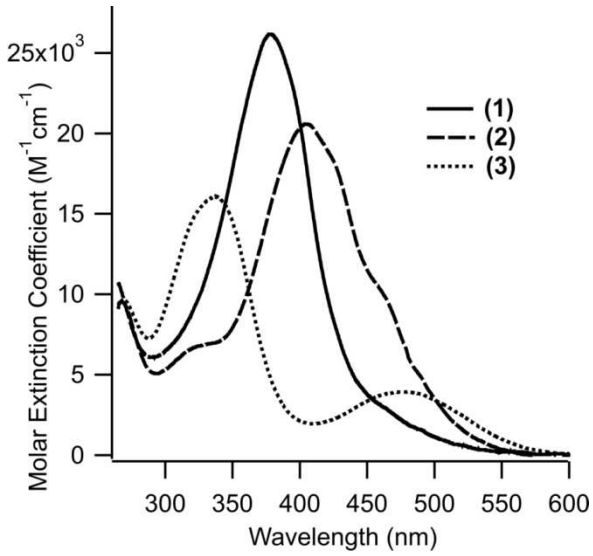


Fig. S1 *Spectra of trans isomers of azobenzene derivatives studied in DMSO, 25°C.*

Table S1. Cis half-lives of azobenzene derivatives studied

Temperature (°C)	$\tau_{½}$ cis-(**1**) in DMSO	$\tau_{½}$ cis-(**2**) in DMSO	$\tau_{½}$ cis-(**3**) in DMSO	$\tau_{½}$ cis-(**3**) in buffer
4	9 hrs	20 hrs	164 days	27 days
25	1.3 hrs	1.9 hrs	14 days	2.4 days
40	25 min	26 min	3 days	12 hours

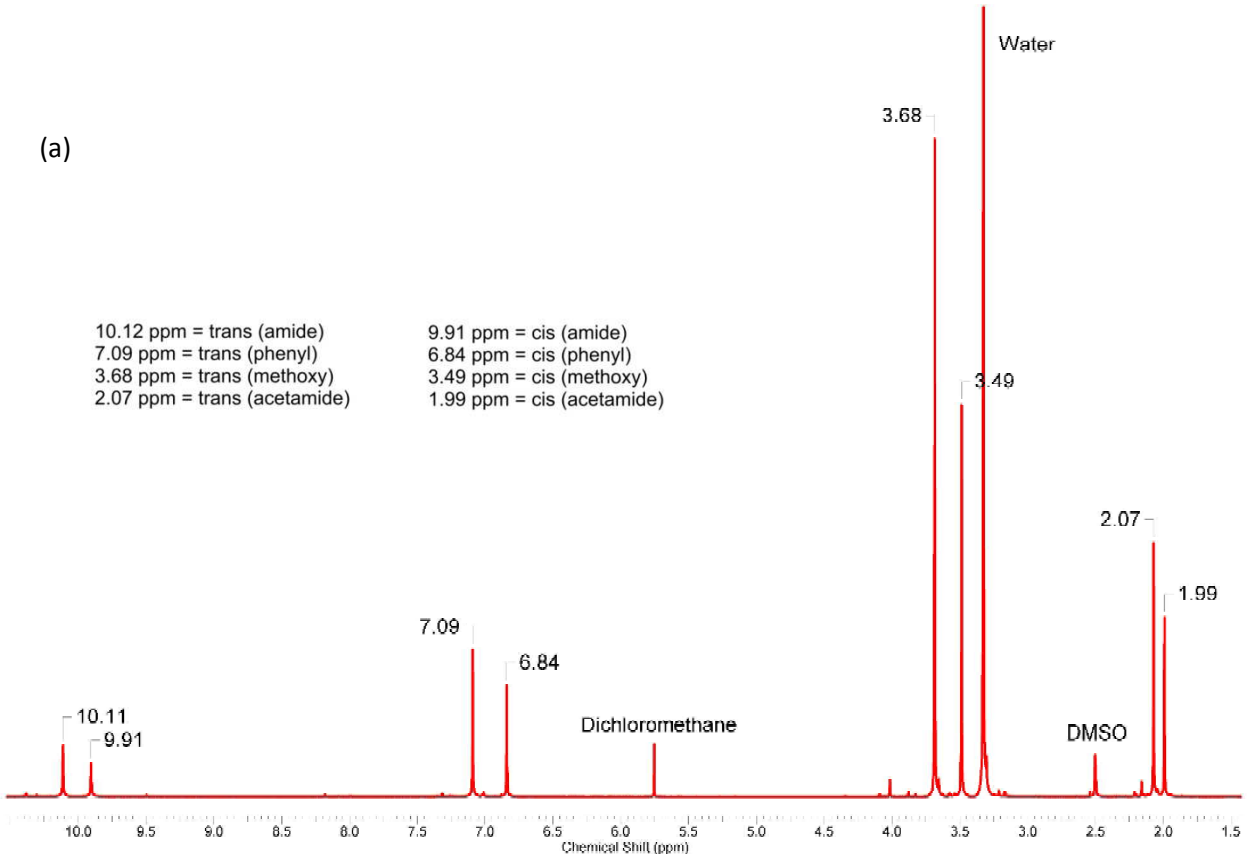
ii) Calculation of cis spectra and extinction coefficients

A concentrated sample of (**3**) in aqueous solution in an NMR tube was irradiated with 370 nm light (897-LZ440U610 LedEngin LED High Power UV LED) for ten minutes while rotating the tube. After that time the sample was quickly transferred to the NMR spectrometer and a 1D proton spectrum was acquired at 25°C. Cis proton chemical shifts appear upfield to those of trans (see NMR spectra Fig. S2(a)). The methoxy protons for each isomer were integrated to calculate the % cis produced. Irradiation was then repeated in exactly the same manner except the sample was diluted and a UV/Vis spectrum was acquired at 25°C. The pure cis spectrum could then be calculated by extrapolation. To calculate the cis spectrum at 4°C, the sample was irradiated at 25°C to generate a known percentage of cis followed by cooling to 4°C where the UV/Vis spectrum was then acquired.

The extinction coefficient was determined by spiking an NMR sample with a known concentration of dichloromethane (10 mM) in DMSO-d_6. The concentration of photoswitch was measured by comparing the area of the methoxy peak with the area of the dichloromethane peak. The photoswitch sample was

then diluted in DMSO or 25 mM sodium phosphate buffer pH 7.0. UV-Vis spectra were acquired to calculate the molar extinction coefficient.

(a)



(b)

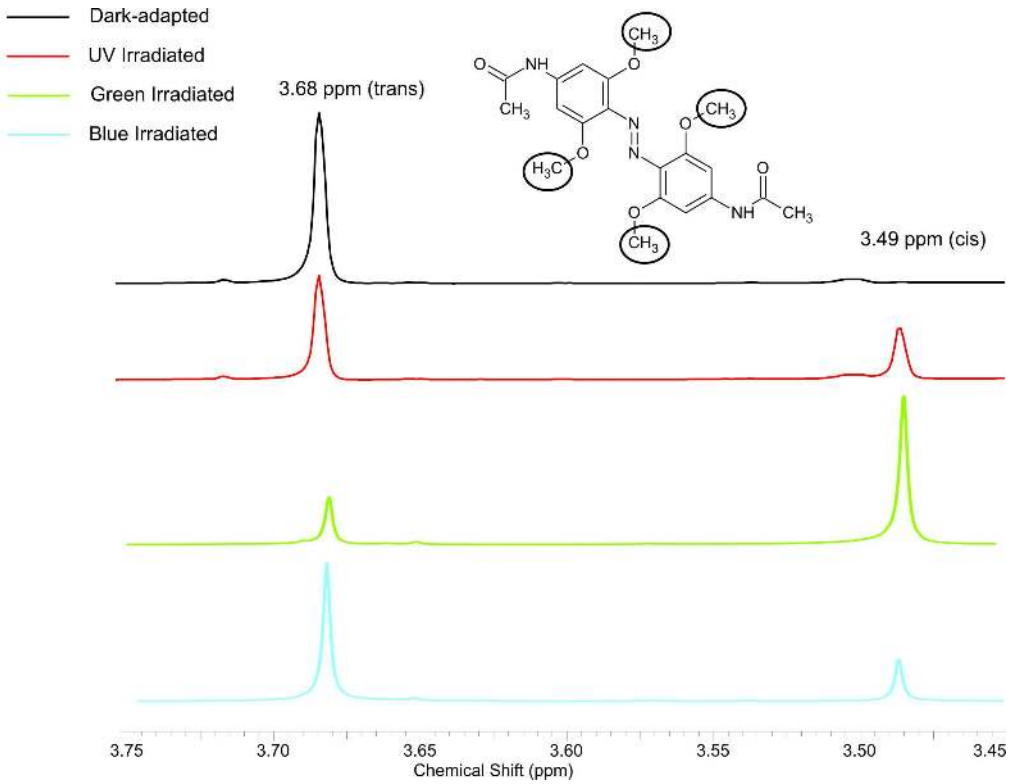


Fig. S2 *Proton NMR spectra of trans and cis (**3**). (a) The full spectrum in DMSO at 25°C acquired after exposure to UV light (365 nm) as described above. (b) Dark-adaptation leads to undetectable amounts of the cis isomer (expansion of the methoxy region). Spectra acquired after green irradiation and blue irradiation are also shown.*

iii) UV/Vis spectra in other solvents

Spectra in methanol, acetonitrile, dichloromethane and dioxane are shown below (Fig. S3). Photoswitching wavelengths and isomer yields observed in the non-polar solvents were similar to those seen in DMSO.

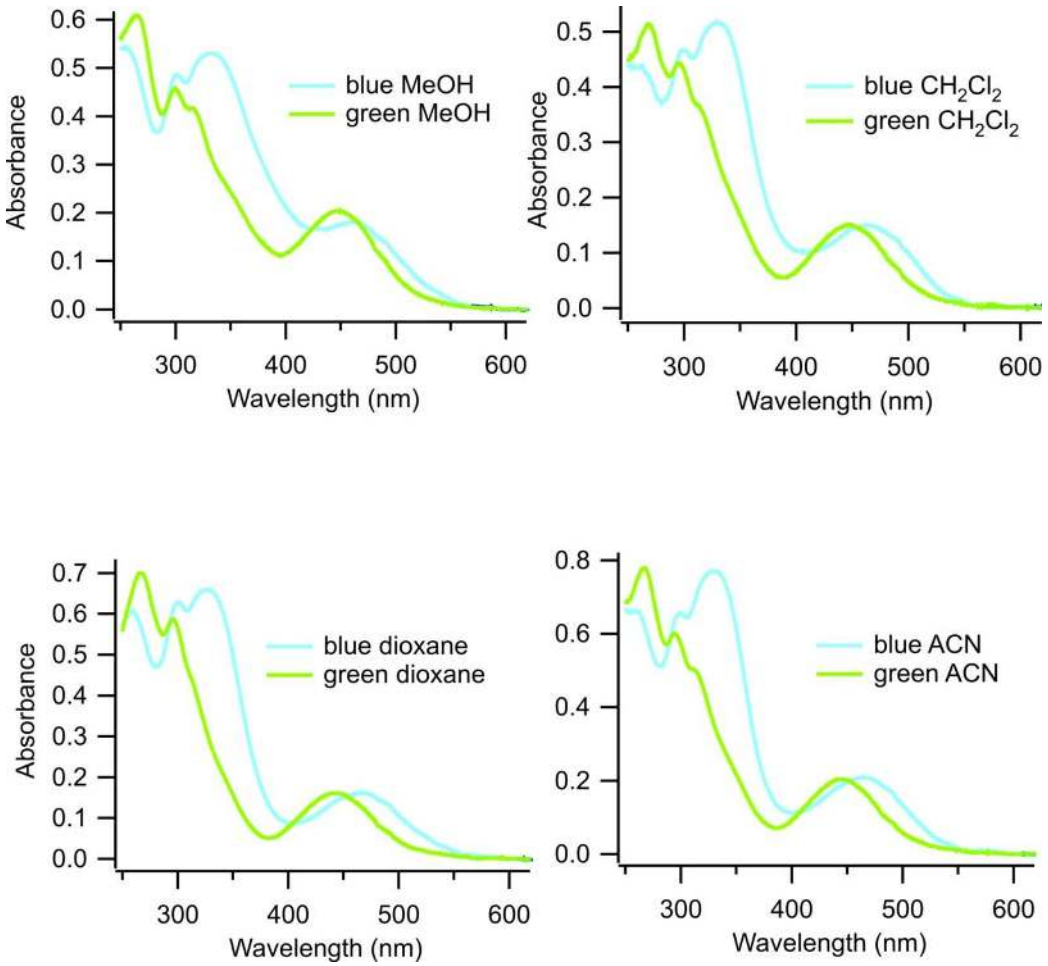


Fig. S3 *Steady state spectra of (**3**) in various solvents (indicated) under green (530 nm) or blue (460 nm) illumination.*

iv) UV/Vis spectra of (3) thermochromism in aqueous solution

The position of the π-π* band of (**3**) was found to be affected by temperature. At higher temperatures the π-π* band of the trans isomer was blue-shifted (compared to that of (**1**)) as it is in DMSO (λ_{max} ~347 nm at 60°C)(Fig. S4 vs. Fig. S5) Conversely, lowering the temperature to 4°C caused a red-shift (λ_{max} ~380 nm) and a hyperchromic effect (Fig. S4).

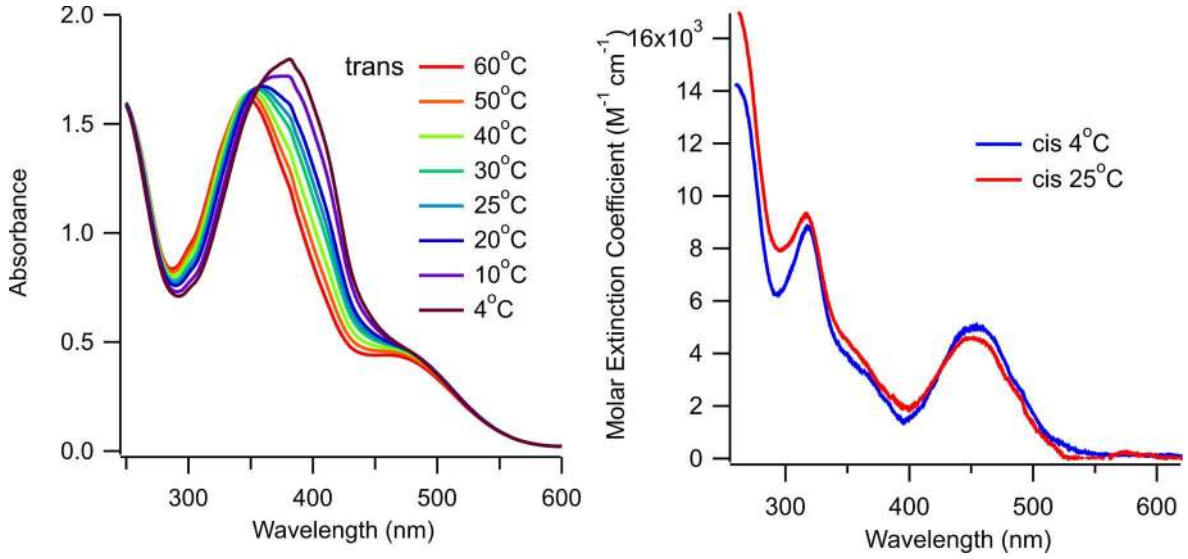


Fig. S4 *UV/Vis spectra of trans-(**3**) (left) and cis-(**3**) (right) (calculated as described above) at a series of temperatures in aqueous solution**.***

It is unlikely that this thermochromism is due to interconversion between two distinct trans conformations (*e.g.* a twisted and planar species) since isosbestic points are not observed.[4] Also, the spectra (at 25°C) do not change appreciably with concentration between 1 and 400 µM implying that self-association is not occurring, or at least, is not changing in this concentration range. Instead, we attribute it to changes in hydrogen bonding between the methoxy groups and water at different temperatures leading to alterations in the effective electronic donating effects of these substituents.[5] This explanation is supported by the lack of thermochromism observed in polar aprotic solvents (*e.g.* DMSO and acetonitrile). Thus, it appears the local environment around the photoswitch in aqueous solution may influence the positions of its absorption bands.

The spectrum of the cis isomer did not appear to be significantly affected by temperature; as a result, there is a smaller separation of the trans and cis n-π* bands in aqueous solution. Nevertheless, irradiation with green wavelengths (530–560 nm) led to production of a large fraction of the cis isomer (~70 %) that could be switched back to the trans form with blue light (~80%) (at 25°C) (Fig. S5) The spectrum of the cis isomer also displayed less solvatochromism (calculated cis spectra in water and DMSO).

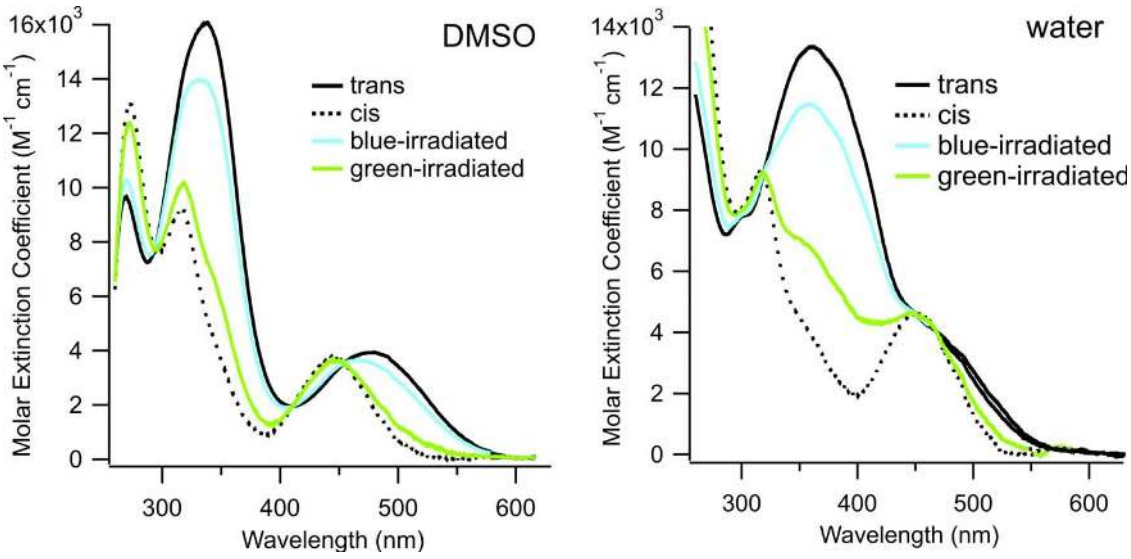


Fig. S5 *Spectra of (**3**) in DMSO (left) and aqueous buffer (right)*

v) UV/Vis spectra of (3) vs pH

The pH dependence of the UV-Vis absorbance spectra in water was investigated. The apparent pKa for the protonated (**3**) species was ~3.8. Spectra were essentially unaffected by pH above pH 5.0.

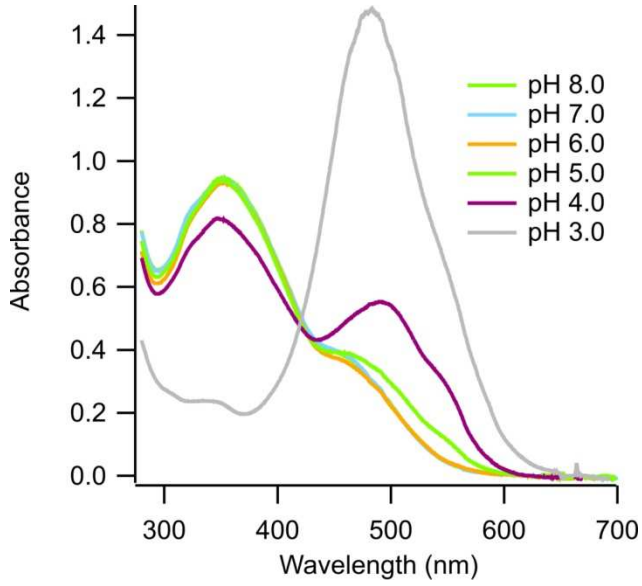


Fig. S6 *Spectra of (**3**) in aqueous buffers of pH indicated.*

vi) Sensitivity to reduction by glutathione

Compound (**3**) was found to be sensitive to reduction by glutathione (the most common reductant in the cellular cytoplasm[6]) in aqueous solution. A stock solution of 0.25 M reduced glutathione (prepared in 0.5 M sodium phosphate buffer pH 7.0) was added to a 15 µM solution of (**3**) in 0.1M sodium phosphate pH 7.0 buffer to give a final glutathione concentration of 1 mM or 10 mM. The final pH of the solution was 7.0. The time course of bleaching was measured by acquiring UV/Vis spectra at 5 minute intervals after addition of glutathione (FigS7(a)). The stability to photoswitching was evaluated by alternately exposing the sample to green (530 nm, Luxeon LXHL-LM5C) blue (460 nm, Luxeon LXHL-LR5C) LED lights in the presence of 1 mM or 10 mM reduced glutathione. (Fig. S7(b)).

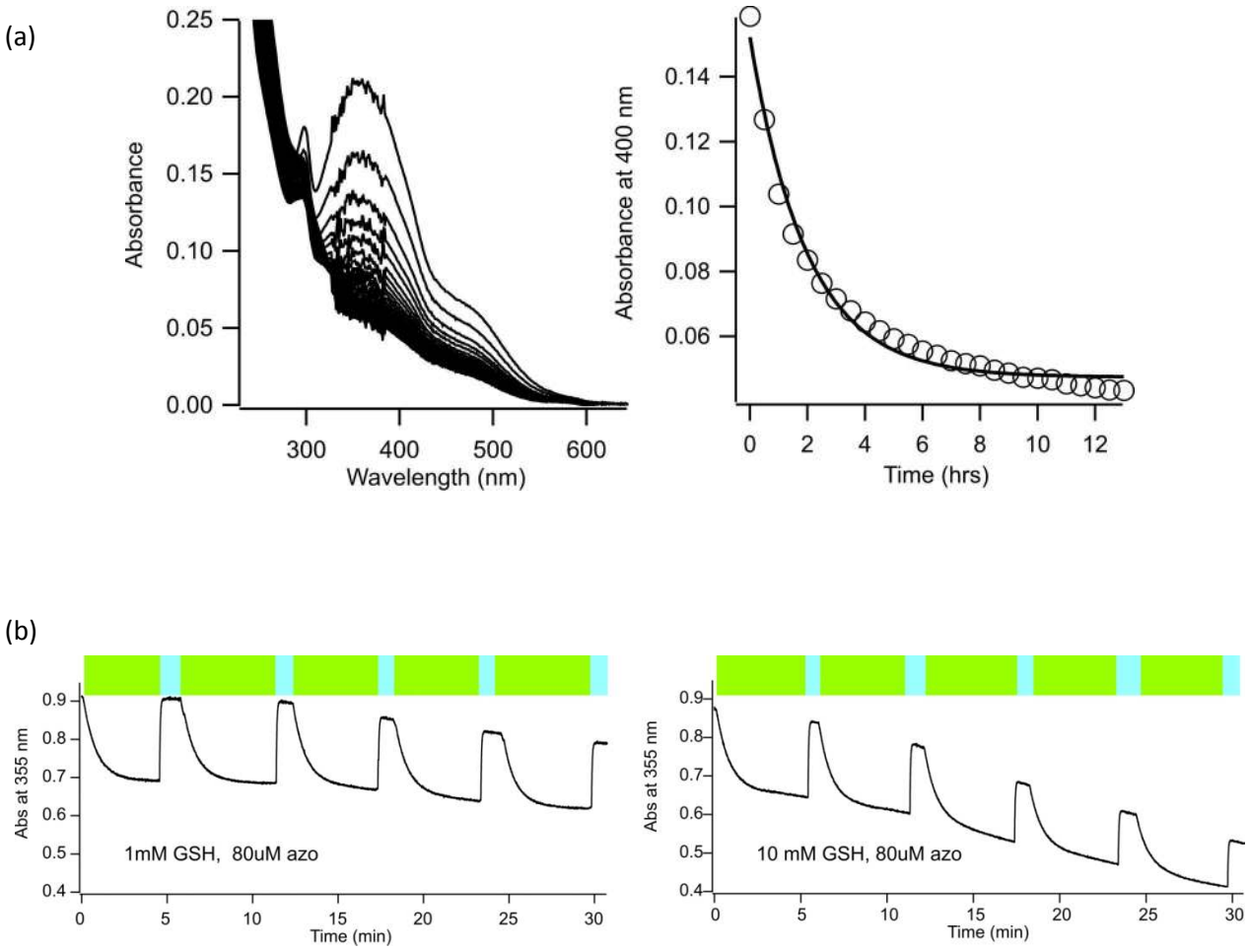


Fig. S7 *(a) Spectra of ~ 15 µM (**3**) in aqueous buffer acquired at 30 min intervals after the addition of 1 mM reduced glutathione. The half life for bleaching is ~1.5 h. (b) Photoswitching of (**3**) at 25°C in aqueous buffer, pH 7.0 containing 1 mM or 10 mM glutathione.*

Molecular Modelling

Conformational searches were performed initially using Hartree-Fock 3-21G methods implemented in Spartan 10 subject to restraints on (trans) amide bonds, terminal methyl, and azo groups which were all pre-minimized at the density functional B3LYP/6-31G* level in vacuum. The top ~50 conformers (of 400) were selected and the energies of each were calculated using DFT B3LYP/6-31G* in vacuum or with DMSO or water solvent treated using the SM8 solvation model.[7] Then, the 5 most stable conformers under each set of conditions were selected and energy minimized using B3LYP/6-31G*. The two most stable conformers vary only in whether the amide carbonyls are pointing the same or opposite directions. Other conformers are mirror images of these or involve slight methyl turns. Structures are shown below. The UV-Vis spectra of the most stable conformers in vacuum and in each solvent were calculated using the TDDFT (B3LYP/6-31G*) methods implemented in Spartan. Tables of transition energies and relative intensities are shown below.

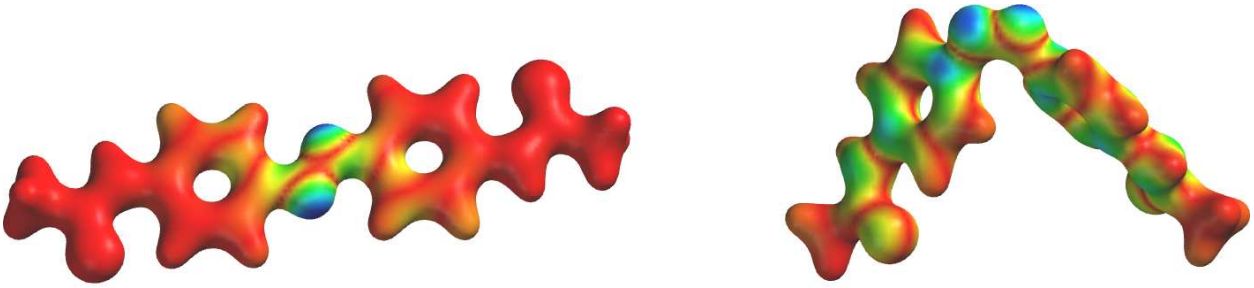


Fig. S8*.Calculated structures for the parent compound (**1**) in DMSO (trans-left and cis-right) with the absolute value of the HOMO-1 for trans (n-π* transition) and HOMO for cis in each case mapped onto the bond density surface. The trans isomer is calculated to be 18.9 kcal/mol more stable than the cis.*

Table S2: *Calculated UV-Vis transitions for (**1**) in DMSO (B3LYP/6-31G*)*

Trans		Cis	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
301.45	0.0124	289.19	0.0003
305.34	0.0001	290.71	0.0001
306.25	0.000006	300.76	0.0117
308.52	0.00001	330.82	0.1162
378.90	1.93	348.43	0.2711
445.57	0.00006	473.42	0.1576

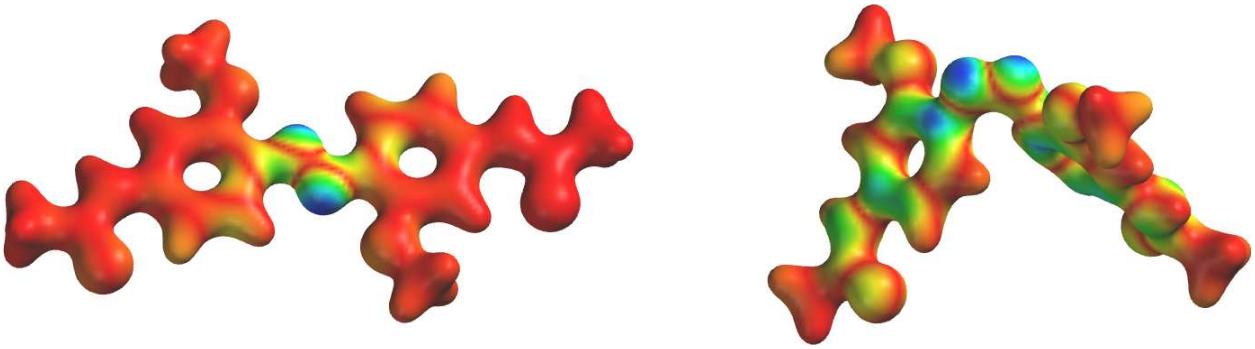


Fig. S9*.Calculated structures for the dimethoxy compound (**2**) in DMSO (trans-left and cis-right) with the absolute value of the HOMO-1 for trans (n-π* transition) and HOMO for cis in each case mapped onto the bond density surface. The trans isomer is calculated to be 18.5 kcal/mol more stable than the cis.*

Table S3: *Calculated UV-Vis transitions for (**2**) in DMSO (B3LYP/6-31G*)*

Trans		*Cis*	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
295.76	0.0001	278.26	0.0013
305.14	0.0023	310.69	0.0331
332.13	0.2184	321.71	0.0547
339.73	0.0097	344.54	0.0771
398.71	1.662	348.54	0.2429
473.66	0.0022	471.58	0.1789

Table S4: *Calculated UV-Vis transitions for (**3**) in DMSO (B3LYP/6-31G*)*

The trans isomer is calculated to be 10.2 kcal/mol more stable than the cis.

Trans		*Cis*	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
270.47	0.0042	267.39	0.1044
310.18	0.0010	326.01	0.2374
335.0	0.0608	330.39	0.0016
336.71	0.0216	339.09	0.0991
348.60	1.2878	347.1	0.048
520.40	0.2187	462.01	0.1401

Table S5: *Calculated UV-Vis transitions for (**3**) in water (B3LYP/6-31G*)*

The trans isomer is calculated to be 8.8 kcal/mol more stable than the cis.

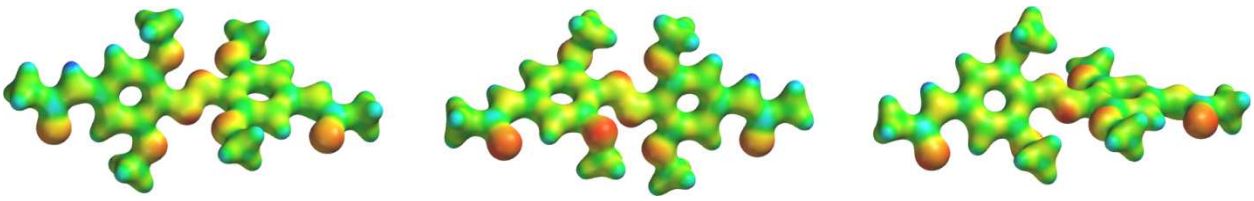
Trans		*Cis*	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
266.45	0.0059	265.22	0.1199
308.85	0.0013	326.07	0.2319
342.52	0.1241	335.2	0.0052
343.75	0.0233	344.38	0.0753
350.79	1.2293	349.14	0.0464
515.14	0.2354	458.43	0.1499

Table S6: *Calculated UV-Vis transitions for (**3**) in vacuum (B3LYP/6-31G*)*

The trans isomer is calculated to be 7.2 kcal/mol more stable than the cis.

Trans min in vac		*Cis min in vac*	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
278.6	0.045	280.6	0.078
318.1	0.004	307.5	0.002
325.7	0.030	309.0	0.185
326.3	0.038	315.2	0.069
342.6	1.229	338.9	0.023
535.7	0.178	441.7	0.095

Models of higher energy conformations (ii),(iii) obtained for trans-(**3**) in water have different conformations of the methoxy substituents compared to the most stable conformer (i). These higher energy trans conformers are ~5-6 kcal/mol more stable than cis. The image below shows the electrostatic potential mapped onto the bond density surface in for each conformer. Red colors indicate areas of higher electron density



(i) (ii) (iii)

Fig. S10*.Calculated structures of trans (**3**) in water. Electrostatic potential is mapped onto the bond density surface in each case.*

Regarding the thermochromism of trans (**3**) in water, the higher energy conformations (i, ii)(Fig. S10) are calculated to have strong transitions at 348 nm and 352 whereas the lowest energy conformer has a transition at 351 nm only. This is consistent with observed blue shifts in the UV (~350 nm) zone as temperature increases.

Table S7: *Calculated* UV-Vis transitions for higher energy conformations of (**3**) in water (B3LYP/6-31G*)

Trans (ii)		*Trans (iii)*	
Wavelength (nm)	Intensity	Wavelength (nm)	Intensity
272.83	0.0063	278.17	0.0065
303.84	0.0696	305.45	0.0808
324.39	0.0115	322.23	0.0165
348.17	0.7588	349.64	0.6518
352.91	0.5445	355.04	0.6808
498.1	0.1937	501.64	0.1876

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