Fast Photoswitches

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Fastest Thermal Isomerization of an Azobenzene for Nanosecond Photoswitching Applications under Physiological Conditions**

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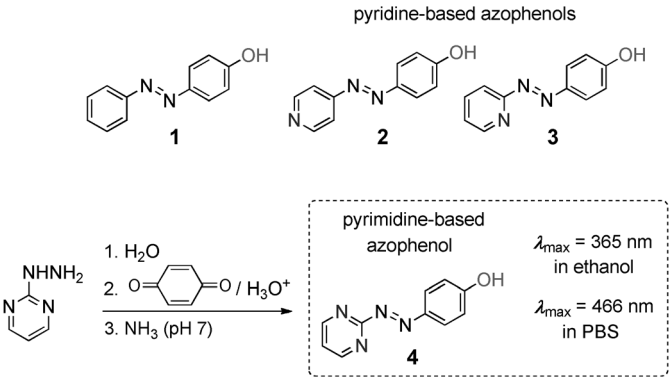
Control of the properties of molecules, for example, their magnetic,[1] electrical,[2] or optical[3] properties, through an external trigger signal is a very interesting design feature of new promising functional systems and smart materials. Indeed, switchable materials have been explored during the last decade within materials science for a wide range of applications, such as micropumps or autonomous valves,[4] photoactive polymers that mimic cilia movement,[5] artificial musclelike actuators,[6] molecular rotary motors,[7] and photo-oscillators.[8] Furthermore, the precise spatial and temporal control of the information transmission of organisms through the application of suitable input energies has become an important topic in cell biology.[9]

Light-responsive materials are a very attractive possibility, since optical triggering has many advantages over both chemical and electrical stimulation. Light is a clean, fast, and environmentally friendly energy source, which can be projected onto a specific position of the target system with great accuracy in a wireless, noninvasive fashion. These useful features have inspired the development of many photoswitching systems whose temporal response range is limited only by the kinetics of the chromophore that acts as a molecular switch.

Azobenzene is doubtless the most widely used organic chromophore for photoswitching applications in biological systems, since it can be successfully incorporated into all types of biopolymers (including peptides, proteins, sugars, and DNA).[10] Azobenzene derivatives can be photoisomerized cleanly to their metastable *cis* form with the appropriate light source in just a few nanoseconds or even more rapidly. Isomerization induces a pronounced change in the optical properties of the system. The reverse process can be induced either by visible light or thermally.[11] Thermal induction of the reverse process is preferred for applications that require real-time information transmission, as it avoids the use of a second optical stimulus for active regeneration of the sample. For this goal to be met, it is essential that the azo dye exhibit a fast thermal *cis*-to-*trans* back conversion, preferably on a sub-microsecond time scale.

Azo dyes that combine a strong push–pull configuration with the ability to establish an azo–hydrazone tautomeric equilibrium are promising chromophores for this purpose, since they show very fast thermal *cis*-to-*trans* isomerization kinetics at room temperature.[12] Specifically, we recently described the use of hydroxyazopiridinium salts for photoswitching applications; these compounds exhibit relaxation times as low as 33 μs at 298 K.[13] Nevertheless, it remains a challenge to increase the isomerization rate of azophenols sufficiently for their isomerization to occur on a sub-microsecond time scale.

Herein we report the synthesis and thermal *cis*-to-*trans* isomerization kinetics of a new highly biocompatible pyrimidine-based azophenol (Scheme 1) that exhibits thermal-



Scheme 1. Chemical structure of azo compounds **1**–**4** and synthesis of azopyrimidine **4**.

relaxation times on the nanosecond time scale at 298 K. The isomerization of this system is 10^3-fold faster than the fastest previously reported azophenol isomerization.[13] Moreover, these fast relaxation times were observed under conditions similar to physiological conditions: a very useful feature for both biological and medical applications. Indeed, azobenzene derivatives have proved useful for diverse applications, for example, as photoswitchable ion-channel blockers,[14] for photocontrol of the activity of enzymes,[15] and in optogenetics,[16] all of which require physiological or life-like conditions.

Scheme 1 shows the different azophenols used in the present study. The kinetics of the thermal *cis*-to-*trans* isomerization process of the different azo derivatives was studied by means of nanosecond laser flash photolysis. We determined

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the relaxation time of the *cis* isomers, τ, by fitting a growing monoexponential function to the data.

4-Hydroxyazobenzene (**1**) showed a relaxation time of 205 ms in ethanol at 298 K (Table 1). To improve the solubility of the azophenol in water and to accelerate the rate of the thermal back reaction, we replaced one of the benzene rings of the azobenzene core with a pyridine ring. The relaxation times of the resulting hydroxy-substituted azopyridines **2** and **3** were lower than that of **1** (14 ms for **2** and 49 ms for **3** in ethanol; Table 1).

Table 1: Relaxation times, τ, for azophenols **1**–**4** in different media.

Azophenol	Medium	T [°C]	τ [s]
1	ethanol	25	205×10^{-3}
2	ethanol	25	14×10^{-3}
3	ethanol	25	49×10^{-3}
4	ethanol	25	6.4×10^{-3}
4	methanol	25	580×10^{-6}
4	acetonitrile	25	201×10^{-3}
4	acetone	25	258×10^{-3}
4	water (pH 6.4)	25	296×10^{-6}
4	water (pH 6.4)	37	203×10^{-6}
4	Tris-HCl buffer, pH 7.4	25	476×10^{-9}
4	Tris-HCl buffer, pH 7.4	37	431×10^{-9}
4	PBS, pH 7.4	25	777×10^{-9}
4	PBS, pH 7.4	37	530×10^{-9}
4	phosphate buffer, pH 7.8	25	40×10^{-9}
4	phosphate buffer, pH 7.8	37	–[a]

[a] Not observed owing to overlap with the laser light scattering.

We decreased the thermal-relaxation time of the photoactive azo dye even further by including a more π-electron deficient pyrimidine ring in the structure (compound **4**, Scheme 1). Azopyridines **2** and **3** and azopyrimidine **4** were all water-soluble as a result of the formation of a hydrogen bond between the nitrogen atoms of the heteroaromatic ring and the surrounding solvent molecules. This phenomenon contributed to the faster isomerization of azopyrimidine **4** in alcoholic media relative to that of azopyridines **2** and **3** (6 ms for **4** versus 14 ms for **2** and 49 ms for **3**), since a greater number of hydrogen bonds can be established with **4**. Moreover, the relaxation time for *cis*-**4** was up to 30-fold faster in water than in ethanol (Figure 1). This result was corroborated by kinetic studies of *cis*-**4** in both acetone and acetonitrile, in which longer relaxation times of 259 and 201 ms were observed, respectively, because of the absence of hydrogen bonding between the azo dye and the solvent molecules.

To further examine the possible use of azopyrimidine **4** for photoswitching purposes in biological probes, the thermal *cis*-to-*trans* isomerization kinetics of *cis*-**4** were investigated in several buffered solutions with pH values ranging from 7.4 to 7.8, which correspond to life-like conditions. Not only in phosphate buffered saline (PBS) but also in Tris-HCl buffer solution (Tris = tris(hydroxymethyl)aminomethane), both at pH 7.4, the thermal *cis*-to-*trans* isomerization reaction took place at 25 °C on the nanosecond time scale, namely, with relaxation times of 777 and 476 ns, respectively (Table 1). A further decrease in the relaxation time to 530 ns in PBS and

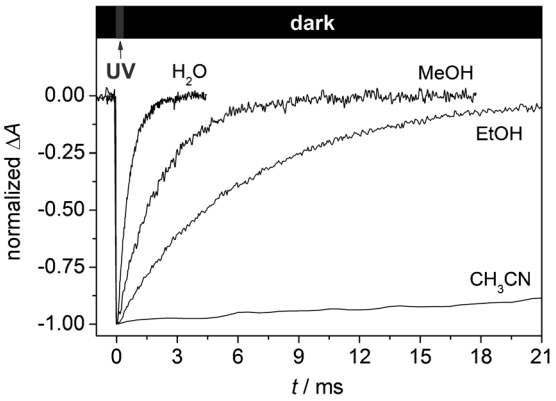

Figure 1. Transient-absorption change photoinduced by laser-pulse irradiation (λ_{irrad} = 355 nm) of the azo dye **4** at 25 °C in different organic solvents ([**4**] = 20 μM, λ_{obs} = 370 nm).

431 ns in Tris-HCl was observed at normal body temperature (37 °C), as could be expected. These fast isomerization kinetics can be explained in terms of the partial deprotonation of the hydroxy group of the molecule at pH 7.4. In both aqueous buffers, the maximum-absorption wavelength for the *trans* azo dye shifted from 365 nm in ethanol to 466 nm, which is very close to the maximum-absorption wavelength of the totally deprotonated azopyrimidine (483 nm).

In continuation of this trend, when the process was studied in a slightly more basic phosphate buffer solution at pH 7.8, the isomerization kinetics were around 20 times faster than that at pH 7.4, with a relaxation time of only τ = 40 ns (Table 1 and Figure 2). Thus, to the best of our knowledge, the hydroxy-substituted azopyrimidine **4** undergoes faster thermal isomerization than any previously reported azo dye.

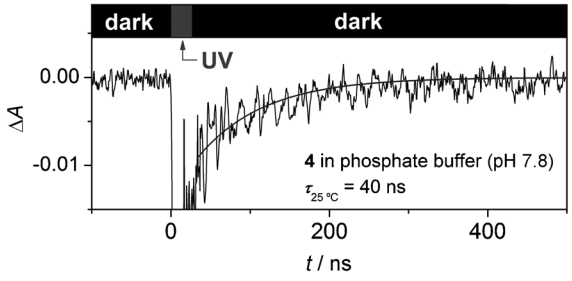

Figure 2. Transient-absorption change photoinduced by laser-pulse irradiation (λ_{irrad} = 355 nm) of the azo dye **4** at 25 °C in a phosphate buffer solution at pH 7.8 ([**4**] = 20 μM, λ_{obs} = 370 nm).

We checked both the repeatability of the isomerization process and the photostability of azopyrimidine **4** under conditions similar to physiological conditions by submitting **4** to thousands of cycles of exposure to pulsed UV light (355 nm, 10 mJ per pulse) and then to darkness. Figure 3 shows the high photostability of the azopyrimidine **4** in a PBS medium at 37 °C: after 50000 cycles, neither the absorbance nor the relaxation time of the system had been altered by the continuous work. Similar behavior was observed for **4** in the different buffer solutions tested.

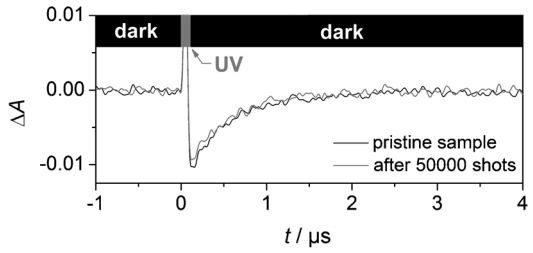

Figure 3. Photostability after 50000 UV-light laser pulses (λ_{irrad} = 355 nm, 10 mJ per pulse) of a solution of the azo dye **4** in PBS under conditions similar to physiological conditions ([**4**] = 20 μM, λ_{obs} = 370 nm, pH 7.4, T = 37 °C).

We tested the biocompatibility of azo dyes **1**–**4** by culturing *Escherichia coli* BL21(DE3) cells with different concentrations of the azo compounds at 37 °C on Luria Broth (LB) plates. A plate without an azo compound was used as a control. We let the cultures grow on the LB plates overnight and then counted the colony-forming units (CFU) on each agar plate (Figure 4). Biocompatibility is expressed as the

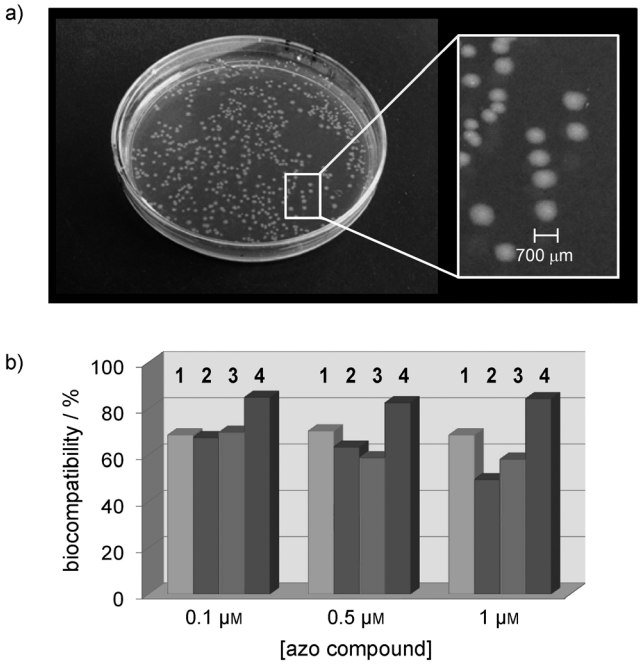

Figure 4. a) *E. coli* BL21 (DE3) colonies cultured at 37 °C on LB plates to which azopyrimidine **4** (10 mM) had previously been added. b) Biocompatibility of azo derivatives **1**–**4** with *E. coli* BL21 (DE3) at different concentrations.

percentage ratio of CFUs on the plate containing the azo compound with respect to those on the control plate. The parent azophenol **1** showed a good biocompatibility of 69% at the three concentrations tested. Azopyridines **2** and **3** also showed a good biocompatibility (around 68%) at 0.1 mM, although it decreased slightly (to 49 and 58%, respectively) when their concentrations were increased to 1 mM. However, azopyrimidine **4** showed a remarkable biocompatibility of 88% at concentrations up to 30 mM, the highest concentration tested. This result clearly indicates the excellent biocompatibility of azopyrimidine **4** even at high concentrations. The low solubility of azo compounds **1**, **2**, and **3** in water precluded the study of their biocompatibility at high concentrations.

In summary, the very short lifetime of the *cis* isomer of azopyrimidine **4** (down to 40 ns at 298 K), its high photostability under conditions resembling physiological conditions, and its excellent biocompatibility even at high concentrations make this photoactive azo dye a valuable chromophore for real-time information-transmission and photonic biological and medical applications.

Experimental Section

Synthesis of **4**: A solution of 2-hydrazinopyrimidine[17] (1.027 g) in water (20 mL) was added slowly to a solution of *p*-benzoquinone (1.026 g) in water (100 mL) and perchloric acid (70%, 6.5 mL). The resulting dark-red solution was stirred for 30 min and then neutralized by the addition of ammonia. The product was obtained by continuous extraction of the mixture with ethyl acetate. Removal of the solvent under reduced pressure gave 2-(4-hydroxyphenylazo)pyrimidine (**4**; 1.289 g, 70%) as a reddish-brown solid. ^{1}H NMR (400 MHz, [D$_6$]acetone, 25 °C): δ = 9.41 (bs, 1H, OH), 8.94 (d, $^3J_{H,H}$ = 4.8 Hz, 2H, ^{Ar}H), 7.95 (d, $^3J_{H,H}$ = 8.8 Hz, 2H, ^{Ar}H), 7.54 (t, $^3J_{H,H}$ = 4.8 Hz, 1H, ^{Ar}H), 7.06 ppm (d, $^3J_{H,H}$ = 8.8 Hz, 2H, ^{Ar}H); ^{13}C NMR (100 MHz, [D$_6$]acetone, 25 °C): δ = 169.6 (1C, ^{Ar}C), 164.2 (1C, ^{Ar}C), 160.8 (2C, ^{Ar}CH), 148.2 (1C, ^{Ar}C), 127.8 (2C, ^{Ar}CH), 122.7 (1C, ^{Ar}CH), 117.9 ppm (2C, ^{Ar}CH); HRMS (ESI): *m*/*z* calcd for $C_{10}H_9N_4O^+$ $[M+H]^+$: 201.0776; found: 201.0774.

Nanosecond laser flash photolysis: A population of *cis* isomers was created by pulsed-laser irradiation of the *trans* isomer at 355 or 532 nm with a Continuum Surelite I-10 Q-switched Nd-YAG laser (pulse width: 5 ns, ca. 10 mJ per pulse). The concomitant absorbance changes were monitored at 90° by a white-light analyzing beam produced by a Xe lamp (PTI, 75 W) in combination with a dual-grating monochromator (PTI 101) coupled to a Hamamatsu R928 photomultiplier for detection.[18]

Biocompatibility tests: Sterile solutions of azo compounds **1**–**4** at concentrations of 0.1, 0.5, and 1 mM as well as 10 and 30 mM in the case of **4** were placed on the surface of LB plates. *E. coli* cells were grown in LB medium (8 mL) overnight, and then a 10^{-4} saline dilution of the cells was prepared. After the absorption of the azo compounds in the LB solid medium, the 10^{-4} *E. coli* cell suspension (100 μL) was placed on agar plates by using the spread plate technique and incubated at 37 °C overnight.

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