Molecular Switches

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Reversible Red Fluorescent Molecular Switches[**]

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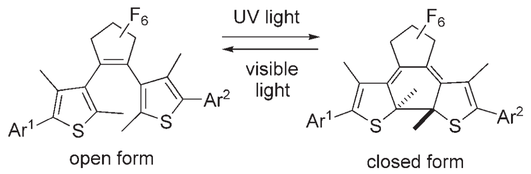
Materials with chemical and physical properties that can be optically modulated are of high interest for new optical recording systems, holographic rewriteable media, drug-delivery applications, biosensors, and advanced microscopic techniques.[1] Photochromic compounds readily lend themselves to this purpose, as they can be reversibly converted by light between two states with different spectroscopic properties.[2] This attribute can then be used to modulate or even switch secondary functions at the molecular or supramolecular level by using light as a trigger.

The reversible optical switching of fluorescence forms the foundation of a new physical concept in lens-based optical imaging to break through the resolution limit caused by diffraction (half the light wavelength).[3] In fact, a resolution at 1/46 of the light wavelength, corresponding to 16 nm, has been achieved with stimulated-emission depletion microscopy, a technique in which the fluorescence of molecular labels is reversibly switched off by ultrafast laser pulses, which induce stimulated emission.[3a] The generalization of this concept, termed RESOLFT (reversible saturable optical fluorescent transitions),[3b,c] showed that the use of ultrafast laser pulses can be avoided if photochromic fluorescent labels are used for the fluorescence switching.[3d] This promising method enables imaging and writing on the nanoscale with ultralow intensities of light. Unfortunately, none of the photochromic compounds synthesized so far possess the required optical properties: pronounced fluorescence modulation, high photostability, and fast switching times. Large fluorescence quantum yields (Φ_{fl}) are also highly desirable, as these allow short imaging times and sensitivity down to a single molecule.

A convenient way to modulate the fluorescence signal of a dye[4] is to attach it to a photochromic compound,[5] either directly[6] or through a linker.[7] The fluorescence modulation is effected through resonant energy transfer (RET)[8] from the fluorescent dye (RET donor) to one of the isomers of the photochromic switch (RET acceptor). For high RET efficiency, the absorption band of the RET acceptor must overlap with the emission band of the donor.

Diheteroaryl ethenes display excellent photochromic properties, can be switched many times, and are chemically and thermally stable. They can be reversibly photoswitched between a colorless open form (OF) and a colored closed form (CF).[5a] The forward reaction is driven by UV light and the reverse reaction by visible light (Scheme 1). Hence, the fluorescence of a fluorophore can be "switched off" when its emission band overlaps the absorption band of the CF of a diheteroaryl ethene, which functions as an RET acceptor.



Scheme 1. Photochromism of 1,2-bis(thiophen-3-yl)cyclopentenes.

To control the photochromic reactions and the excitation of the fluorophore specifically, a large separation between the absorption bands of the OF, the CF, and the fluorophore is preferred. However, most of the fluorescent switches reported so far[9] do not fulfill this condition. Most compounds with a high degree of fluorescence modulation have only moderate or low Φ_{fl} values;[6d–f,h,j,n] in some cases, these values have not been reported.[6a–c,i,k,l,7a,b,d] Others possess a relatively low fluorescence modulation, especially when the ring-closing reaction is not activated at short wavelengths (254 or 313 nm).[6c,d,7b] Hence, their use in biological microscopy is precluded by the high autofluorescence background and expected photodamage. Moreover, aberration-corrected lenses with high numerical apertures (1.35–1.45) are only available for wavelengths greater than 360 nm. The goal of the present study was to synthesize photoswitchable fluorescent compounds with substantially improved performance in the visible or near-UV wavelength range. Particular importance was placed on increasing the fluorescence modulation at longer switching wavelengths and at the same time obtaining high Φ_{fl} values. For this purpose, we had to find a robust photochromic compound and a photo- and chemically stable fluorescent dye, and connect the two with a linker that enables effective RET between the donor and acceptor.

Rhodamine dyes are known to be good fluorescent labels that have large absorption coefficients and fluorescent quantum yields and show high photostability and a low rate of triplet formation. Moreover, they are preferred in biological applications because their excitation band between 550 and 600 nm results in lower autofluorescence compared with excitation in the UV or blue region. Owing to its high degree of substitution, the planar molecule rhodamine 101 (Rh 101) is one of the most stable fluorophores with Φ_{fl} close to unity.[10a] Its carboxy group is sterically hindered, and thus coupling

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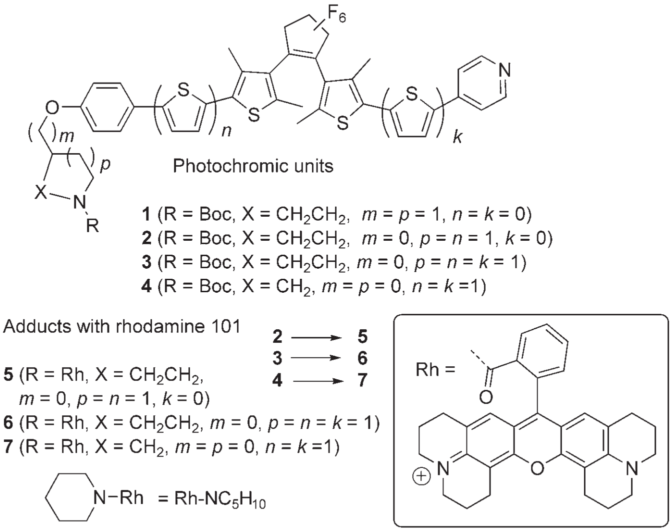
reactions with amines require special activation (the amino group is a convenient functional group for attachment to the fluorophore).[10b] The acylation of piperidine (chosen as a prototype for the linker) with Rh 101 to give compound Rh-NC_5H_{10} results in a red shift of the absorption band from 560 to 583 nm and the emission band from 589 to 604 nm without significant change in Φ_{fl} (Tables 1 and 2). This compound was prepared to study the effect on the fluorophore of substitution, and to evaluate the fluorescence of Rh-NC_5H_{10} in the absence of RET. The emission band of Rh-NC_5H_{10} required a photochromic system with an absorption maximum at about 620–640 nm for the CF.

Table 1: Photochemical and photophysical properties of the building blocks **1**–**3** and Rh-NC_5H_{10}.[a]

Compd	λ_{max} [nm] (ε×10^{-4} [$M^{-1}cm^{-1}$]) OF	CF	α_{PS}[b]	Φ OF→CF[b]	CF→OF	R_0 [Å]
1	277 (3.0)	578 (1.4)	0.88	0.28	8.2×10^{-3}[c]	44
2	342 (2.4) 308 (2.3)	605 (1.5)	0.95	0.065	7.3×10^{-4}[c]	49
3	345 (4.1)	640 (1.9)	0.96	0.10	2.5×10^{-4}[d]	51
Rh-NC_5H_{10}	583 (10)[e]	–	–	–	–	

[a] Absorption coefficients (ε) were taken from Figure 1, the degree of conversion in the photostationary state (α_{PS}) was measured by HPLC, and quantum yields for the photochromic reactions ($\Phi_{OF\to CF}$ and $\Phi_{CF\to OF}$) were calculated from irradiation experiments.[14] Förster radii (R_0) for RET between the corresponding CF and the fluorophore (Rh-NC_5H_{10}) were calculated from the spectral properties of the components (see Figure 1).[16] Errors are approximately 10% for the quantum yields and absorption coefficients, and 2% for the conversion in the photostationary state. [b] Irradiation: 313 nm. [c] Irradiation: 577 nm. [d] Irradiation: 610 nm. [e] Emission maximum: 604 nm.

As a starting point, we chose a system described by the group of Lehn.[11] It consists of a 1,2-bis(2-methyl-3-thienyl)perfluorocyclopentene core with 4-hydroxyphenyl group at the 5-position of one thiophene ring and 4-pyridyl substituent at the 5′-position of the other thiophene ring. Its OF and CF in benzene absorb at 296 and 602 nm, respectively. The degree of conversion into the CF in the photostationary state (α_{PS}) was reported to be greater than 98% (in benzene). To increase the photochemical stability of this system, we blocked each of the unsubstituted thiophene positions with a methyl group[12] to prepare the photochromic compound **1** (Scheme 2). The major absorption maxima of the OF and the CF are at 277 and 578 nm, respectively (in EtOH). To shift the absorption of the CF to longer wavelengths, we inserted a thiophene ring between the pyridyl residue or the phenoxy group and the central core, and thus synthesized compounds **2** and **3** with an extended π system. Extended π conjugation is known to increase the ε value of the closed isomer and improve the conversion into the closed form, owing to a decrease in the quantum yield of the ring-opening reaction.[13] The linker units were synthesized through a Mitsunobu reaction of available *N*-Boc-4-hydroxy(methyl)piperidines with 4-iodophenol.[14] The precursor with a shorter linker (**4**) was obtained from 2-phenoxyethyl amine.[14] The carboxy group of Rh 101 was activated with the peptide coupling reagent HATU, and the adducts **5**–**7** were isolated in good yields. The final compounds were purified by HPLC where necessary.



Scheme 2. Photochromic units **1**–**4** and their adducts with rhodamine 101 (**5**–**7**). Boc = *tert*-butyloxycarbonyl.

The spectroscopic and photochromic properties of the novel molecular switches **5**–**7** and the building blocks **1**–**4** and Rh-NC_5H_{10} were studied in ethanol by irradiating diluted solutions (ca. 10^{-5} M) with light of different wavelengths.[14] The properties of the building blocks are summarized in Figure 1 and Table 1. The conversion of the parent photochromic compound **1** was incomplete (α_{PS} = 88% at 313 nm), which is disadvantageous as the highest fluorescence modulation achievable is limited by the conversion of the photochromic unit. We define the fluorescence modulation (FM) as $1-F_{PS}/F_0$, in which F_0 and F_{PS} are the fluorescence signals of the initial and the photostationary (PS) states, respectively. The addition of one or more thiophene rings increases the

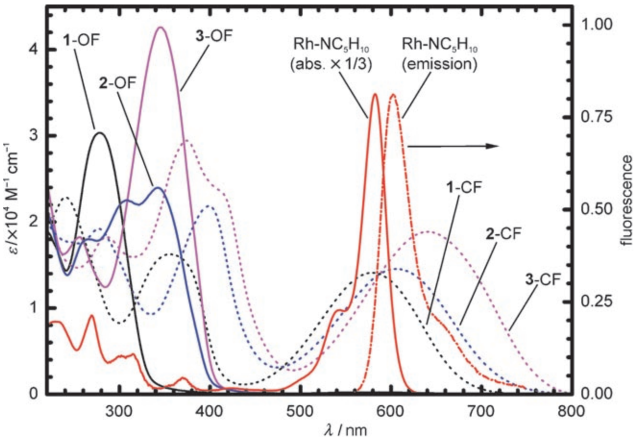


Figure 1. Absorption spectra (left axis) of the photochromic compounds **1**–**3**; open forms (OF) are plotted with full lines and closed forms (CF) with dotted lines (**1**: black, **2**: blue, **3**: purple). Absorption spectrum (solid red line) and fluorescence emission (dotted red line, right axis) for the fluorescent building block Rh-NC_5H_{10} are also shown.

value of α_{PS} up to nearly 100 %. The photocyclization reaction (OF→CF) was performed at 313, 366, and 375 nm, and the values of α_{PS} were found to be independent of the irradiation wavelength. A red shift and a slight increase in the absorption coefficient is observed in the absorption spectra of the CF with increasing number of thiophene rings. The addition of one thiophene ring in the OF (**2**) leads to splitting into two absorption bands, both of which are red-shifted relative to the spectrum of **1**. Compound **3** has only one major band at approximately the same wavelength as the second band of **2** (345 nm). The addition of thiophene units decreased the quantum efficiency of the ring-opening reaction ($\Phi_{CF\rightarrow OF}$), but no clear pattern was observed for the photocyclization reaction ($\Phi_{OF\rightarrow CF}$). The spectroscopic and photochromic properties of compounds **3** and **4** were identical, despite the different linkers involved; therefore, compound **4** is not included in Table 1 and Figure 1.

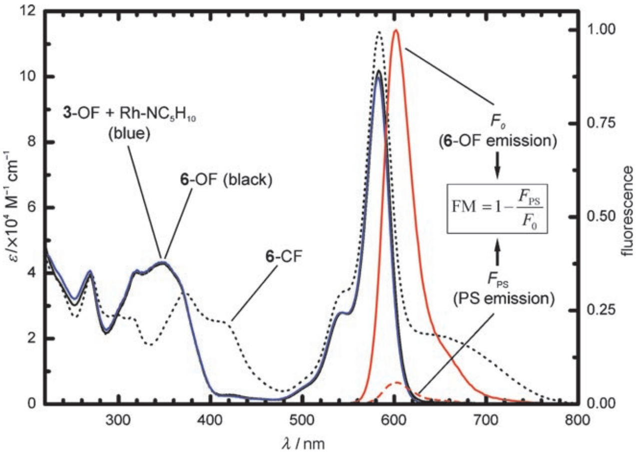


Figure 2. Absorption spectra (left axis) of **6** (solid black line: OF, dotted black line: CF) and the sum of the absorption spectra of compounds **3** and Rh-NC_5H_{10} (blue line). Fluorescence emission (right axis) of **6** in the OF (solid red line) and in the PS state under irradiation at 375 nm (dashed red line, 98 % CF); the different intensities demonstrate the fluorescence modulation.

Table 2: Photochemical and photophysical properties of the fluorescent switches **5**–**7**.[a]

Compd	Φ		Φ_{fl}	α_{PS}[b]	FM	τ_{fl} [ns]	E_{RET}[12]	
	OF→CF[b]	CF→OF[c]					E_{OF}	E_{CF}
Rh-NC_5H_{10}	–	–	0.97	–	–	4.5	–	–
5	0.032	5.5×10^{-4}	0.72	0.98	0.86	3.3	0.26	0.91
6	0.063	2.2×10^{-4}	0.72	0.98	0.94	3.3	0.26	0.97
7	0.061	2.2×10^{-4}	0.29	0.98	0.92	1.3	0.72	0.98

[a] The fluorescence modulation (FM), as well as $\Phi_{OF\rightarrow CF}$ and $\Phi_{CF\rightarrow OF}$ values were calculated from irradiation experiments. α_{PS} was measured by HPLC,[14] and Φ_{fl} was measured by stationary methods.[15] Fluorescence lifetimes (τ_{fl}) are given for the OF.[14] See the Supporting Information for details of the calculation of E_{OF} and E_{CF}.[14] Errors are approximately 10% for quantum yields and absorption coefficients, 5% for fluorescence lifetimes, and 2% for the conversion in the photostationary state and fluorescence modulation. [b] Irradiation: 313 nm. [c] Irradiation: 660 nm.

The photochromic building blocks **2**–**4** were attached to Rh 101, whereas compound **1** was disregarded because of its lower α_{PS} value (< 0.9). A shorter linker was also used (**4**) to study the effect of a decrease in the distance between the donor and the acceptor. The properties of the final adducts (**5**–**7**) are summarized in Table 2. All the absorption spectra indicate the absence of perturbations due to the binding of donor and acceptor units. The spectra of **6** are given in Figure 2 as an example. The blue line is the sum of the spectra of the components **3**-OF and Rh-NC_5H_{10}. It is identical within experimental errors to the spectrum of **6**-OF (Figure 2, black line). Moreover, the emission spectra of all compounds (**5**–**7** and Rh-NC_5H_{10}) are identical, and a decrease in $\Phi_{OF\rightarrow CF}$ values was observed for each of the final adducts relative to the corresponding photochromic building blocks **2**–**4**. This tendency can be attributed to the fact[7c] that some of the irradiation light is absorbed by the fluorophore and hence cannot contribute to photocyclization. The efficiencies of the reverse reaction ($\Phi_{CF\rightarrow OF}$) are nearly unaffected by coupling to Rh 101. The values obtained for α_{PS} were slightly enhanced and are close to 100 % for all adducts (**5**–**7**).

FM, Φ_{fl}, fluorescence lifetimes (τ_{fl}), and RET efficiencies of both isomers (E_{OF} and E_{CF}) were measured in dilute solutions (optical density < 0.05) to avoid any self-absorption effects.[15] Two important conclusions follow from the results (Table 2): First, comparison of **5** and **6** reveals an increase in FM and E_{CF}. This result correlates with the calculated R_0 value (Table 1), which is slightly larger for **3**, the building block of **6**. Second, a shorter linker results in only a slightly larger E_{CF} value for compound **7** relative to **6**, but a significantly larger E_{OF} value. This increase is responsible for the poorer FM value of **7**. Consequently, a longer linker may be introduced to reduce E_{OF} while leaving E_{CF} unchanged. In other words, the linker may be lengthened without problem to insert a binding unit for attaching the whole fluorescent structure as a label to a substrate.

In summary, we have prepared switchable fluorescent compounds with improved properties in the visible wavelength region which make them suitable for application in information storage and fluorescence microscopy. The photocyclization reaction responsible for quenching the fluorescent signal can be activated by focusable light. The synthesized compounds **5**–**7** possess large fluorescence modulation (> 85 %) even with irradiation at 375 nm, where many photostable diaryl perfluorocyclopentenes do not absorb.[5a] The photocyclization reaction of these switches can be carried out with laser diodes. Large RET efficiencies for the energy transfer between the fluorophore and the CF of the photochromic moieties account for the strong fluorescence modulations observed. All compounds have good fluorescence quantum yields (≥ 30 %), in particular **5** and **6** (70 %). The fluorescence signal can be probed at 550–600 nm and detected between 600 and 700 nm, thereby minimizing the generation of background signal. Further work involves attaching reactive groups to **6** in order to bind it to polymer precursors, nanoparticles, and biomolecules. As a label in a RESOLFT method, this or similar compounds could boost the spatial resolution of far-field fluorescence microscopy by at least an order of magnitude.

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[9] Outstanding fluorescence modulation was reported for condensed dithieno(thiophenes)[6a,b] or ethinyl-Bodipy[6i] residues as fluorophores directly attached to 1,2-bis(2-alkyl-3-thienyl)perfluorocyclopentenes. The highest conversion to the closed form was 92 %[6a,b] and 96 %[6i] and was achieved at 365 and 254 nm, respectively. In both cases, the remaining fluorescence was 10–11 %. When anthracene was connected through a CH_2 group to an acceptor, 87 % conversion to the nonfluorescent CF was observed at the PS state at 355 nm.[7a] Irie and co-workers[7c] used the 1,3-adamantane linker and studied in detail the single molecule properties of the highly fluorescent 2,5-dimethoxy-bis(9,10-phenylethinyl)anthracene. Similar fluorophores connected to two acceptor units displayed around 80 % fluorescence modulation and 83 % Φ_{fl}.[6g] In another study,[7b] in which Lucifer yellow was connected to different acceptors through relatively long and flexible cadaverine linkers acylated with succinic or butanoic acid residues, the fluorescence decreased by 65 or 84 % in the PS state (at 313 and 320 nm, respectively).

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