Lighting Molecules with Light: Fluorescence Activation with Photochromic Oxazines

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LIGHTING MOLECULES WITH LIGHT: FLUORESCENCE ACTIVATION WITH PHOTOCHROMIC OXAZINES

By

Janet Cusido

A DISSERTATION

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LIGHTING MOLECULES WITH LIGHT: FLUORESCENCE ACTIVATION WITH 
PHOTOCHROMIC OXAZINES

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Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength and then emit after irradiation at an exciting wavelength. The interplay of such activation and excitation events can be exploited to switch fluorescence on in a defined region of space at a given interval of time. In turn, the spatiotemporal control of fluorescence translates into the opportunity to implement imaging and spectroscopic schemes that are not possible with conventional fluorophores. Specifically, photoactivatable fluorophores permit the monitoring of dynamic processes in real time as well as the reconstruction of images with subdiffraction resolution. These promising applications can have a significant impact on the characterization of the structures and functions of biomolecular systems. As a result, strategies to implement mechanisms for fluorescence photoactivation with synthetic fluorophores are particularly valuable. In this context, the work described in this thesis explores operating principles to activate the emission of organic chromophores with the aid of photochromic auxochromes. Specifically, I synthesized molecular dyads combining fluorescent and photochromic components within their covalent skeleton. These photoswitchable compounds all have an oxazine as their photochromic component and differ in the nature of their fluorescent component. In particular, I incorporated
arene, borondipyrromethene (BODIPY), coumarin, cyanine, and oligothiophene fluorophores in these dyads and characterized their photochemical and photophysical properties with a combination of steady-state and time-resolved spectroscopic measurements. The compound integrating a coumarin-oxazine fluorophore turned out to be the dyad with the most spectroscopic signature for the acquisition of fluorescence images with subdifraction resolution. As a result, I developed also hydrophilic derivatives of this compound as well as a supramolecular strategy to impose hydrophilic character on it. The latter protocol is based on the encapsulation of the photoactivatable compound within the hydrophobic interior of polymer nanoparticles. Indeed, in collaborative studies, we demonstrated that these nanoscaled assemblies can be resolved in space even when they are separated by subdifraction distances on the basis of fluorescence photoactivation.
TO MY LOVED ONES
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CHAPTER 1

PHOTOACTIVATABLE FLUOROPHORES

1.1. Background

Certain organic molecules emit light in the form of fluorescence upon excitation at an appropriate wavelength.\(^1\) This behavior offers the opportunity to probe biological structures and processes with optical methods.\(^2\) Indeed, labeling strategies to introduce fluorescent probes within biological samples, in combination with microscopic techniques to excite the labels and collect their fluorescence, permit the noninvasive visualization of a diversity of specimens in real time.\(^3\) In particular, the basic operating principles of a fluorescence microscope (Figure 1.1) involve the illumination of the labeled specimen with an excitation source through an objective lens.

![Diagram of a fluorescence microscope](image_url)

**Figure 1.1.** The fluorescence microscope records images of samples labeled with fluorescent molecules by exciting the labels and collecting their emission.

The radiation focused on the sample excites the many fluorescent labels from their ground state (\(S_0\) in Figure 1.2) to one of their excited singlet states (e.g., \(S_2\)). The excited species relax thermally to the first singlet excited state (\(S_1\)) and then radiatively to \(S_0\). The emitted light is collected on the detector, through the very same objective lens, to
reconstruct an image of the labeled sample with micrometer resolution on a millisecond timescale. In fact, the fluorescence microscope has become an indispensable analytical tool in the biomedical laboratory for the routine investigation of cells and tissues.

**Figure 1.** The excitation of a fluorescent molecule from the ground state \((S_0)\) to the second singlet excited state \((S_2)\) is followed by internal conversion to the first singlet excited state \((S_1)\). The molecule in \(S_1\) can either decay nonradiatively or emit light in the form of fluorescence to regenerate \(S_0\). Alternatively, it can undergo intersystem crossing to populate the first triplet excited state \((T_1)\) and then either decay nonradiatively or emit light in the form of phosphorescence to regenerate \(S_0\).

The power of fluorescence microscopy in permitting the visual inspection of biological samples revolves around the ability of individual fluorescent probes to absorb exciting radiation and emit as a result.\(^1\) Thus, the identification of viable strategies to manipulate the photophysical properties of organic molecules is central to the further development of this discipline. In particular, mechanisms to switch molecules from a nonemissive to an emissive state under the influence of optical stimulation allow the photoactivation of fluorescence. Interest in such photoactivatable fluorophores was sparked initially by the idea of monitoring the course of photochemical reactions with fluorescence measurements\(^4\) and then by the possibility of using such compounds for photographic applications.\(^5\) It eventually became apparent, however, that photoactivatable fluorophores can be a valuable complement to conventional ones in a diversity of imaging applications.\(^6\)–\(^12\) Indeed, fluorescence photoactivation permits the monitoring of dynamic processes in real time\(^13\)–\(^24\) as well as the acquisition of images with spatial resolution at the nanometer level.\(^25\)–\(^42\) As a result, significant research efforts are
presently directed to the development of strategies for fluorescence photoactivation with fluorescent proteins and synthetic dyes together with their implementation in imaging applications. In fact, the former can conveniently be encoded genetically and permit the visualization of living specimens.\textsuperscript{17–22} The latter can be engineered with superior photochemical and photophysical properties, relying on the aid of chemical synthesis\textsuperscript{6–9,12}, and their small dimensions, relative to their natural counterparts, ensure minimal steric perturbations to labeled samples.\textsuperscript{43} In this chapter, I review the basic principles governing the photophysical properties of organic molecules, highlight the main mechanisms developed so far to photoactivate fluorescence with synthetic chromophores, illustrate representative examples of photoactivatable synthetic probes and discuss their promising applications.

1.2. Photophysical Properties of Organic Molecules

An organic molecule can adopt a finite number of electronic configurations with distinct energies.\textsuperscript{44} These electronic states differ in the distribution mode of the many electrons among the available molecular orbitals and their energy separations approach the energies associated with ultraviolet and visible radiation. As a result, a molecule can gain a sufficient amount of energy to change its electronic configuration by absorbing an ultraviolet or a visible photon.

The electronic configuration with the lowest possible energy (ground state) of a neutral organic molecule distributes pairs of electrons with opposite spin in the half of available molecular orbitals with lowest energy. Therefore the sum (c) of the individual electronic spins is equal to 0 in the ground state. This value corresponds to a multiplicity ($2c + 1$) of 1 and, hence, this particular electronic state is a singlet and is indicated with the notation $S_0$ (Figure 1.2).

Upon absorption of a photon with appropriate energy, an electron in one of the occupied molecular orbitals moves to one of the unoccupied molecular orbitals. During this
transition, the electron retains its spin and the multiplicity of the overall system does not change. Thus, the resulting electronic state (excited state) is also a singlet, even although it has two unpaired electrons.

Distinct singlet excited states can be accessed from the very same ground electronic configuration. Indeed, a molecule in the initial state $S_0$ has multiple pairs of occupied and unoccupied molecular orbitals available to permit electronic transitions with different energies. Therefore, excitation from $S_0$ can populate any one of the accessible singlet excited states, depending on the energy of the absorbed photon. For example, the diagram in Figure 1.2 shows a transition from $S_0$ to the second singlet excited state ($S_2$) upon excitation.

The electronic transition that accompanies the absorption of a photon from $S_0$ occurs on a femtosecond timescale. During this extremely fast process, the nuclear configuration of the molecule does not change and the geometry adopted in $S_0$ is retained in the final electronic state (e.g., $S_2$ in Figure 1.2). After the transition, however, the nuclei respond to the change in electronic configuration by adjusting their relative positions. As a result, the excited molecule alters its geometry to release a fraction of the energy gained with excitation and, eventually, changes its electronic configuration to decay nonradiatively to $S_1$. This relaxation process, called internal conversion (Figure 1.2), occurs generally on a timescale of picoseconds. Once $S_1$ is populated, the excited molecule can return back to $S_0$ by either releasing thermal energy or emitting light in the form of fluorescence. The decay from $S_1$ to $S_0$ tends to occur over times ranging from hundreds of picoseconds to a few nanoseconds.

The two unpaired electrons of a molecule in $S_1$ have opposite spin. One of them can change its spin to produce an electronic state with a total spin ($\sigma$) of 1 and a multiplicity $(2\sigma + 1)$ of 3. Therefore, this particular state is a triplet and is indicated with the notation $T_1$. The process responsible for the transformation of $S_1$ into $T_1$ is called intersystem
crossing (Figure 1.2), occurs generally over several nanoseconds and is followed by either the release of thermal energy or the emission of light in the form of phosphorescence to populate S\textsubscript{0}. The transformation of T\textsubscript{1} into S\textsubscript{0}, however, requires one of the two unpaired electrons to change its spin and happens over times ranging from few nanoseconds to several microseconds.

Photochemical reactions also offer the opportunity to release the excitation energy of a molecule.\textsuperscript{45} Specifically a compound in S\textsubscript{1} can undergo a structural transformation to produce a different species, which can then decay to its ground electronic configuration. Thus four distinct relaxation pathways are available to deactivate S\textsubscript{1}, but only one of them is responsible for fluorescence. Indeed S\textsubscript{1} can (1) decay to S\textsubscript{0} thermally, (2) decay to S\textsubscript{0} radiatively, (3) intersystem cross to T\textsubscript{1} or (4) undergo a photochemical reaction. As a result, the quantum efficiency of fluorescence is ultimately dictated by the rates of these competitive processes.\textsuperscript{1} In particular the fluorescence quantum yield (\(\Phi_F\)) is the ratio between the number of emitted photons (\(N_E\)) and the number of the absorbed photons (\(N_A\)) and is related to the rate constants of fluorescence (\(k_F\)), nonradiative decay (\(k_{NR}\)), intersystem crossing (\(k_{ISC}\)) and photochemical conversion (\(k_{PC}\)), according to equation (1). Therefore the ability of a fluorophore to emit light can, in principle, be controlled by manipulating these four rate constants with appropriate structural modifications.

\[
\Phi_F = \frac{N_E}{N_A} = \frac{k_F}{k_F + k_{NR} + k_{ISC} + k_{PC}}
\]  

(1)

1.3. Mechanisms and Structural Designs for Irreversible Fluorescence Photoactivation

Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength (\(\lambda_{Ac}\) in Figure 1.3). The photogenerated species is then irradiated at an exciting wavelength (\(\lambda_{Ex}\) in Figure 1.3) to emit light in the form of fluorescence. This behavior can be implemented with two general mechanisms.\textsuperscript{6–9,12} One of them acts on the ability of S\textsubscript{0} to absorb the exciting radiations and the other
controls the nonradiative deactivation of $S_1$. In the first instance, the switchable system is designed to absorb at $\lambda_{\text{Ex}}$ only after the transformation induced by $\lambda_{\text{Ac}}$. Under these conditions, activation enables the population of the excited state responsible for fluorescence. In the second instance, the initial species can also absorb at $\lambda_{\text{Ex}}$, but nonradiative decay dominates its relaxation. After the transformation induced by $\lambda_{\text{Ac}}$, $k_{\text{NR}}$ is designed to decrease significantly, relative to $k_F$, in order to enhance $\Phi_F$, according to equation (1).

Figure 1.3. Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength ($\lambda_{\text{Ac}}$) and then emit upon irradiation at an exciting wavelength ($\lambda_{\text{Ex}}$).

Both mechanisms for fluorescence activation can be implemented by integrating switchable and fluorescent components within the same covalent skeleton.\textsuperscript{6–9,12} The local excitation of the former can then be achieved with an activating beam operating at $\lambda_{\text{Ac}}$ to induce the cleavage of one or more covalent bonds. This photolytic process separates irreversibly the two components and, in some instances, is also followed by a rearrangement or a supramolecular event. Ultimately, the photoinduced structural transformation either enables the absorption of the fluorescent fragment at $\lambda_{\text{Ex}}$ or suppresses the nonradiative decay of its excited state. This general design logic is extremely versatile and can easily be adapted to most of the main families of fluorescent probes with the aid of chemical synthesis. Indeed, photoactivatable acridinone,\textsuperscript{46} anthracene,\textsuperscript{47} benzofurazan,\textsuperscript{48} borondipyrromethene,\textsuperscript{49} chromone,\textsuperscript{50} coumarin,\textsuperscript{51–56} dihydrofuran,\textsuperscript{57–59} fluorescein,\textsuperscript{60–67} naphthalene,\textsuperscript{68} pyranine,\textsuperscript{69} pyrazolines,\textsuperscript{70} resorufin,\textsuperscript{71} rhodamine,\textsuperscript{66,72–76} thioxanthone\textsuperscript{77} and xanthone\textsuperscript{78} derivatives have already been developed successfully on the basis of these operating principles. Furthermore, this general strategy
has been adapted to activate also the luminescence of conjugated oligomers\textsuperscript{79} and semiconductor quantum dots.\textsuperscript{80,81}

The behaviors of compounds 1\textit{a}\textsuperscript{57} and 2\textit{a}\textsuperscript{64} (Figure 1.4) are representative examples of the two main mechanisms for fluorescence photoactivation. The former has a photocleavable azide group attached to a dihydrofuran fluorophore. The latter has a photocleavable nitrobenzyl group attached to a fluorescein fluorophore. The absorption spectrum of 1\textit{a} in ethanol shows a band centered at 424 nm.\textsuperscript{57} Upon illumination at a $\lambda_{Ac}$ of 407 nm, the azide group cleaves to generate irreversibly 1\textit{b}. This structural transformation shifts the absorption band to 570 nm. Thus, irradiation at a $\lambda_{Ex}$ (594 nm) positioned within this band can excite exclusively the photogenerated species 1\textit{b}. It follows that the fluorescence of this molecule is observed only after activation at $\lambda_{Ac}$ and excitation at $\lambda_{Ex}$.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure14.png}
\caption{Illumination of 1\textit{a} at an activating wavelength ($\lambda_{Ac}$) cleaves the azide group to generate 1\textit{b} and enable absorption at an exciting wavelength ($\lambda_{Ex}$). Irradiation of 2\textit{a} at a $\lambda_{Ac}$ cleaves the nitrobenzyl appendage to produce 2\textit{b} and discourage nonradiative decay upon excitation at a $\lambda_{Ex}$. Both processes translate into irreversible fluorescence activation.}
\end{figure}

The absorption spectrum of 2\textit{a} in neutral buffer reveals an intense band in the visible region for the heterocyclic chromophore.\textsuperscript{64} Upon illumination at $\lambda_{Ex}$ of 490 nm, 2\textit{a} changes its electronic configuration to populate $S_1$. However, the transfer of one electron
from the phenoxy appendage to the heterocyclic fragment encourages the nonradiative deactivation of $S_1$. As a result, the fluorescence quantum yield of $2a$ is negligible. Upon irradiation at a $\lambda_{Ac}$ of 350 nm, the nitrobenzyl group attached to the heterocyclic chromophore cleaves to generate $2b$. The anionic character of the photogenerated species prevents intramolecular electron transfer in $S_1$ and favors, instead, the radiative decay of this state. In fact, illumination of $2b$ at $\lambda_{Ex}$ is accompanied by significant emission. Thus, also for this particular system, fluorescence is observed only after activation at $\lambda_{Ac}$ and excitation at $\lambda_{Ex}$.

The irreversible photolysis of the azide group of $1a$ and nitrobenzyl appendage of $2a$ releases an intact fluorescent fragment that can then emit upon excitation at an appropriate wavelength.$^{57,64}$ In some instances, however, the released fragment must associate with a supramolecular partner in order to discourage nonradiative decay and enable emission.$^{67,68}$ In other instances, the photochemical reaction does not release the intact fluorophore and, instead, generates an intermediate species that spontaneously rearranges into the final fluorescent fragment.$^{50,52,54,56}$ Alternatively, such photolytic events can be coupled with energy transfer to activate fluorescence.$^{65,66}$ For example, compound $3a$ (Figure 1.5) pairs a coumarin donor to a fluorescein acceptor within its covalent skeleton.$^{65}$ The nitrobenzyl appendage attached to the donor cleaves upon illumination at a $\lambda_{Ac}$ of 365 nm in neutral buffer. This structural transformation shifts bathochromically the absorption of the coumarin fragment and enables its excitation at a $\lambda_{Ex}$ of 410 nm. The excited donor can then transfer energy to the fluorescein acceptor, which then emits at 520 nm. Thus, the fluorescence of the acceptor is eventually activated through the manipulation of the absorption properties of the donor.
Figure 1.5. Illumination of 3a at an activating wavelength ($\lambda_{Ac}$) cleaves the nitrobenzyl group to generate 3b and enable the local excitation of the coumarin donor upon irradiation at an exciting wavelength ($\lambda_{Ex}$). The process is followed by the transfer of energy to the fluorescein acceptor and, ultimately, results in the activation of the fluorescence of the latter.

1.4. Mechanisms and Structural Designs for Reversible Fluorescence Photoactivation

The operating principles for fluorescence activation associated with 1a–3a are centered around the photolysis of a covalent bond.\textsuperscript{57,64,65} This photoinduced process is irreversible and, therefore, the fluorescence of the photochemical product cannot be switched off after activation. Reversible fluorescence activation can, instead, be achieved relying on the inherent reversibility of photochromic transformations.\textsuperscript{82–87} Indeed, photochromic compounds switch from a colorless to a colored state under illumination at an appropriate wavelength and return to the original form either thermally or upon irradiation at another wavelength. In fact, the photoinduced coloration of a photochromic compound is, essentially, imposing a bathochromic shift on the absorption spectrum similar to that associated with the interconversion of 1a into 1b. It follows that the photogenerated state of the photochromic component can, eventually, be excited selectively at an appropriate $\lambda_{Ex}$. If this species is fluorescent, then its emission is observed after activation at a $\lambda_{Ac}$ designed to trigger the photochromic transformation and illumination at $\lambda_{Ex}$ to excite the photogenerated state.\textsuperscript{89–94} This behavior is identical to that associated with the photoinduced conversion of 1a into 1b except for the fact that the photochromic process is reversible. Therefore, the original nonemissive state can be regenerated and the system can undergo multiple fluorescence activation/deactivation cycles. Nonetheless, the
fluorescence quantum yields of most photochromic compounds are fairly modest, relative to those of the main families of fluorescent probes, with the exception of certain diarylethenes,\textsuperscript{95–97} rhodamines\textsuperscript{98–102} and spiropyrans.\textsuperscript{103,104}

The behaviors of compounds \textit{4a}\textsuperscript{98} and \textit{5a}\textsuperscript{97} (Figure 1.6) are representative examples of reversible fluorescence activation on the basis of photochromic transformations. The former has a lactame ring connected to a xanthene heterocycle through a spirocenter. Upon illumination at a $\lambda_{Ac}$ of 366 nm in a poly(vinyl alcohol) matrix, the [C–N] bond at the spirocenter cleaves to generate the rhodamine \textit{4b}. This transformation is accompanied by a significant bathochromic shift in absorption. In fact, the photogenerated isomer can be excited selectively with a $\lambda_{Ex}$ of 530 nm. The diarylethene \textit{5b} has three adjacent [C=C] bonds at its core. Upon irradiation at a $\lambda_{Ac}$ of 313 nm in dioxane, they undergo an electrocyclic rearrangement to close a six membered ring in the form of compound of \textit{5b}. Once again, this structural transformation imposes a significant bathochromic shift in absorption and the photogenerated isomer can be excited selectively at a $\lambda_{Ex}$ of 488 nm. Thus, both systems offer the opportunity to photoactivate fluorescence with a mechanism that is essentially identical to that governing the behavior of \textit{1a}. In these instances, however, the photogenerated species \textit{4b} and \textit{5b} can switch back to the original nonemissive states \textit{4a} and \textit{5a} respectively, offering the opportunity to perform multiple activation/deactivation cycles. Nonetheless, \textit{4b} is thermally unstable, while \textit{5b} is thermally stable. The former reverts spontaneously to \textit{4a} on a millisecond timescale and the latter converts to \textit{5a} photochemically under visible illumination.
Figure 1.6. Illumination of 4a and 5a at an activating wavelength ($\lambda_{Ac}$) encourages the formation of 4b and 5b respectively. The photogenerated isomers can be excited selectively with irradiation at an appropriate exciting wavelength ($\lambda_{Ex}$) to emit light in the form of fluorescence. Both transformations are reversible. The original nonemissive state 4a is regenerated thermally, while 5a is reformed photochemically.

The photochromic transformations of 4a and 5a result in the formation of a fluorescent chromophore that was not originally present in the initial structure.$^{97,98}$ Alternatively a preformed fluorophore can be connected to an appropriate photochromic component and the photoinduced interconversion of the latter can be exploited to control the emission of the former.$^{89-94}$ Indeed, numerous examples of fluorophore–photochrome dyads have already been developed successfully, relying on this general design logic.$^{105-127}$ In most of these systems, however, the photogenerated state of the photochromic component is engineered to quench the emission of the fluorescent partner on the basis of either electron or energy transfer. As a result, the behavior of these functional compounds translates into fluorescence deactivation. Instead in this dissertation, I report the synthesis and properties of a family of dyads specifically designed for fluorescence activation. In these compounds, the photochromic component regulates reversibly the ability of the fluorescent fragment to absorb at $\lambda_{Ex}$ and permits the implementation of an activation mechanism similar to that governing the behavior of 1a.
1.5. Photochemical and Photophysical Parameters Associated with Fluorescence Activation

The operating principles behind most photoactivatable fluorescent probes revolve around the coupling of a photochemical reaction (activation) with a photophysical process (fluorescence). The first of these two steps requires the nonemissive species to absorb photons at $\lambda_{Ac}$ and switch to the emissive state. As a result, the molar extinction coefficient ($\varepsilon_{Ac}$) of the former at $\lambda_{Ac}$ and the quantum yield ($\Phi_P$) for this photochemical transformation should be as large as possible. The second step demands the emissive state to absorb photons at $\lambda_{Ex}$ and emit light in the form of fluorescence. Thus, the molar extinction coefficient ($\varepsilon_{Ex}$) of this species at $\lambda_{Ex}$ and $\Phi_F$ should also be as large as possible. As representative examples, the values of these parameters for 1a–5a are listed in Table 1.1.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\lambda_{Ac}$ (nm)</th>
<th>$\varepsilon_{Ac}$ (mM$^{-1}$ cm$^{-1}$)</th>
<th>$\Phi_P$</th>
<th>$\lambda_{Ex}$ (nm)</th>
<th>$\varepsilon_{Ex}$ (mM$^{-1}$ cm$^{-1}$)</th>
<th>$\Phi_F$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a/b</td>
<td>407</td>
<td>29</td>
<td>0.0059</td>
<td>594</td>
<td>54</td>
<td>0.025</td>
<td>57</td>
</tr>
<tr>
<td>2a/b</td>
<td>350</td>
<td>—</td>
<td>0.03</td>
<td>490</td>
<td>—</td>
<td>0.86</td>
<td>64</td>
</tr>
<tr>
<td>3a/b</td>
<td>365</td>
<td>28</td>
<td>0.3</td>
<td>410</td>
<td>80</td>
<td>—</td>
<td>65</td>
</tr>
<tr>
<td>4a/b</td>
<td>366</td>
<td>—</td>
<td>—</td>
<td>530</td>
<td>—</td>
<td>—</td>
<td>98</td>
</tr>
<tr>
<td>5a/b</td>
<td>313</td>
<td>17</td>
<td>0.42</td>
<td>488</td>
<td>46</td>
<td>0.87</td>
<td>97</td>
</tr>
</tbody>
</table>

[a] The reported parameters were measured in EtOH for 1a/b, neutral buffer for 2a/b and 3a/b, poly(vinyl alcohol) for 4a/b and dioxane for 5a/b. $\varepsilon_{Ac}$ and $\varepsilon_{Ex}$ were determined at the wavelengths corresponding to the maxima of the corresponding absorption bands, rather than at the actual $\lambda_{Ac}$ and $\lambda_{Ex}$. The missing data were not reported in the original articles.

Compounds 2a–5a have acceptable $\varepsilon_{Ac}$ and $\Phi_P$ values to switch to the respective emissive counterparts at moderate activation intensities.\textsuperscript{64,65,97,98} However, they require a $\lambda_{Ac}$ in the ultraviolet region. In fact, most of the photoactivatable fluorophores developed so far have essentially the very same limitation. Indeed, biological samples have plenty of intrinsic chromophores able to absorb in the ultraviolet region and, therefore, can co-
absorb activating radiations in this wavelength range with harmful consequences. This problem can be overcome with structural modifications of the photocleavable group aimed at shifting its absorption from the ultraviolet to the visible region. For example, the introduction of two methoxy substituents on a nitrobenzyl group can be exploited to enable activation with wavelengths longer than 400 nm. Alternatively, two-photon absorption processes can be invoked to induce activation. Under these conditions, the simultaneous absorption of two near-infrared photons can encourage the transformation of the nonemissive species into the emissive one. Such protocols offer also the excellent penetration depths and spatial resolutions inherent to two-photon excitation schemes. Nonetheless, they are limited to photocleavable groups with sufficiently large two-photon absorption cross sections.

Compounds 1b–5b can all be excited with a λ_{Ex} in the visible region and have all a relatively large ε_{Ex}. Both conditions generally apply to most of the photoactivatable fluorophores developed so far for biological applications. Instead, Φ_{F} tends to vary over a broad range of values. The Φ_{F} of 1b, for example, is only 0.025, while that of 5b is up to 0.87. Nonetheless, a modest Φ_{F} is often compensated by a large ε_{Ex} and the product of the two (brightness) for 1b is sufficiently large to permit the detection of this fluorophore at the single-molecule level.

In addition to the wavelengths, molar extinction coefficients and quantum yields listed in Table 1.1, two additional parameters contribute to define the performance of a photoactivatable fluorophore. One of them is the ratio (contrast) between the emission intensity of the emissive state and that of the nonemissive one. Ideally, the nonemissive state should not fluorescence upon illumination at λ_{Ex}. In a photoactivatable system operating on the basis of changes in the rate of nonradiative decay, however, the nonemissive state can also absorb at λ_{Ex} and the partial radiative deactivation of its excited state might contribute some fluorescence. For example, 2a absorbs at λ_{Ex} and emits with a Φ_{F} close to 0.001. After activation and the formation of 2b, Φ_{F} increases
to 0.86 with a significant enhancement in detected fluorescence. Nonetheless, the minimal fluorescence contribution of the nonemissive state in such systems can have detrimental effects on contrast and might limit the use these probes in imaging applications requiring negligible background signal.

The other parameter of significance is the photobleaching resistance of the emissive state. The population of the S\(_1\) of this species, upon excitation at \(\lambda_{\text{Ex}}\), can be followed by intersystem crossing to T\(_1\). Both S\(_1\) and T\(_1\) can undergo photochemical transformations that result in the bleaching of the emissive species. These processes are generally not particularly efficient but, over the course of multiple excitation cycles, degradation can become significant. It follows that photoactivatable fluorophores with limited ability to resist photobleaching might not be particularly useful in imaging applications requiring prolonged excitation.

1.6. Applications of Photoactivatable Fluorophores

The unique combination of photochemical and photophysical properties of photoactivatable fluorophores translates into an opportunity to control the spatial and temporal distribution of fluorescence. As a result, these functional fluorescent probes permit the implementation of imaging and spectroscopic protocols that are otherwise inaccessible with conventional fluorophores. Indeed, photoactivatable fluorophores are routinely used as calibration standards in photolysis experiments,\(^\text{128–130}\) to probe the activity and dynamics of biomolecular systems,\(^\text{48,49,55,61,62,68,71,131–139}\) to monitor the flow dynamics of fluids\(^\text{140–149}\) and to reconstruct subdiffraction images.\(^\text{57–59,74–76,98,99,103,104,150–157}\)

The common theme of all these strategies is the interplay of activating and exciting beams to switch fluorescence on within a defined region of space at a given interval of time.

The ability to activate fluorescence under optical control can be exploited to monitor the diffusion of molecules in a diversity of media.\(^\text{13–24}\) In fact, the dynamics of a
photoactivatable fluorophore within a substrate of interest can be probed by irradiating at $\lambda_{Ac}$ a portion of the sample and at $\lambda_{Ex}$ the entire substrate, while measuring the intensity of the emitted light. This illumination protocol permits the monitoring of the temporal change in the spatial distribution of the activated fluorescence. For example, the photoinduced transformation of the nonemissive species $6a$ (Figure 1.7) into the emissive one $6b$ offers the opportunity to follow the diffusion of the latter in glycerol on a millisecond timescale.$^{158}$ Specifically, the tip of an optical fiber can be positioned on a glycerol drop containing $6a$ to illuminate a relatively small area of the sample at a $\lambda_{Ac}$ of 365 nm. These irradiation conditions cleave the two nitrobenzyl groups to generate $6b$ within the illuminated area. The simultaneous irradiation of the whole substrate at a $\lambda_{Ex}$ of 490 nm reveals fluorescence mostly within the activated area ($a$ in Figure 1.7). However, images recorded with the same illumination protocol after a delay of 400 and 900 ms ($b$ and $c$ in Figure 1.7) show clearly a significant widening of the fluorescent area. Thus, the photogenerated species $6b$ is diffusing away from the activated area over the course of this temporal scale.

![Diagram](image)

**Figure 1.7.** Illumination of $6a$ at an activating wavelength ($\lambda_{Ac}$) of 365 nm cleaves the two nitrobenzyl groups to generate $6b$, which then emits upon irradiation at an exciting wavelength ($\lambda_{Ex}$) of 490 nm. As a result, a sequence of images of a glycerol drop containing $6a$, recorded exciting at $\lambda_{Ex}$, reveal fluorescence only after illumination at $\lambda_{Ac}$. Specifically, pulses at $\lambda_{Ac}$ applied after 100 ($a$), 500 ($b$) and 1000 ($c$) ms from the onset of the frame sequence to the central region of the imaging field activate fluorescence. Interestingly, the fluorescent area widens over time indicating that the activated probes diffuse over this temporal scale. The drawing in $a$ represents the tip of the optical fiber used to deliver the activating radiation. The scale bar in $b$ corresponds to 10 $\mu$m.
The illumination protocol to monitor diffusion with photoactivatable fluorophores is conceptually related to fluorescence recovery after photobleaching (FRAP).\textsuperscript{159–161} However, FRAP demands high irradiation intensities to bleach fluorophores and switch emission off in the illuminated area. By contrast, moderate illumination intensities are generally sufficient to switch photoactivatable fluorophores and, therefore, they can conveniently be adapted to monitor diffusion in biological specimens.\textsuperscript{13–24} In a seminal study, for example, the dynamics of actin microfilaments could be visualized on a timescale of seconds with the aid of a photoactivatable resorufin.\textsuperscript{71} Specifically, one of cystein residues of actin can be connected covalently to a photoactivatable label in the form of the conjugate 7a (Figure 1.8). Illumination of 7a at a $\lambda_{AC}$ of 360 nm cleaves the nitrobenzyl group to generate 7b, which can be excited at a $\lambda_{Ex}$ of 575 nm to emit light in the form of fluorescence. The injection of 7a into goldfish epithelial keratocytes results in its incorporation within the native actin microfilaments. Illumination at $\lambda_{Ac}$ of a defined area within a labeled keratocyte activates fluorescence. Sequences of images (a–c in Figure 1.8), recorded upon irradiation at $\lambda_{Ex}$ over the course of several seconds, clearly reveal a change in the spatial distribution of the activated fluorescence that is indicative of microfilament dynamics. In fact, similar imaging protocols have been extended to the investigation of the dynamics of a diversity of biomolecular systems.\textsuperscript{131,132,135–139}
Figure 1.8. Illumination of 7a at an activating wavelength ($\lambda_{Ac}$) of 360 nm cleaves the nitrobenzyl group to release 7b, which can be irradiated at an exciting wavelength ($\lambda_{Ex}$) of 575 nm to emit light in the form of fluorescence. The injection of 7a in goldfish epithelial keratocytes results in the incorporation of this conjugate within intracellular actin microfilaments. Images of the labeled sample, recorded exciting at $\lambda_{Ex}$ 4 (a), 81 (b) and 136 s (c) after illumination at $\lambda_{Ac}$ of a portion of a keratocyte, show a spatial redistribution of fluorescence over time. This change is indicative of microfilament dynamics. The scale bar in a corresponds to 10 µm.

In addition to the diffusion of individual molecules within a media of interest, similar configurations can be adapted to the investigation of fluid dynamics.\textsuperscript{140–149} In particular, a solution of a photoactivatable fluorophore flowing through the channels of a microfluidic device can be probed, once again, on the basis of activation and excitation events. For example, a dilute solution of 6a in basic buffer can be introduced within a rectangular channel of micrometer dimensions fabricated within a poly(dimethyl siloxane) matrix.\textsuperscript{147} Illumination at a $\lambda_{Ac}$ of 337 nm of a fixed position within the channel cleaves the two nitrobenzyl groups of 6a to generate its emissive counterpart 6b. The activated species travels along the channel to reach, eventually, an area that is illuminated at a $\lambda_{Ex}$ of 488 nm. The fluorescence emitted from the excited area can be measured simultaneously to build a temporal profile of the number of detected photons. The resulting plots show that photons are detected after a delay from activation that is directly related to the flow rate. Indeed, an increase in flow rate from 11 cm s$^{-1}$ to 16 m s$^{-1}$ results in a decrease in delay from milliseconds to microseconds (a and b in Figure 1.9).
Figure 1.9. Illumination of a solution of 6a flowing through a microscaled channel with an activation source generates 6b. The emissive species travels through the channel to reach an area irradiated with an excitation source. The emitted photons are detected at this position and their number is plotted against the delay from activation. The resulting profiles show a decrease in delay with an increase in flow rate from 11 cm s\(^{-1}\) (a) to 16 m\(\text{s}^{-1}\) (b).

The phenomenon of diffraction limits the lateral resolution of the fluorescence microscope to hundreds of nanometers.\(^2\) As a result, individual fluorophores can be resolved in space only if their distance is significantly greater than their physical dimensions. It follows that this convenient imaging technique cannot appreciate the subtle factors that govern biological processes and define biological structures at the molecular level. These stringent limitations can be overcome with the aid of photoactivatable fluorophores.\(^{26-42}\) Indeed, the ability to switch fluorescence on under optical control permits the separation of distinct probes in time and the sequential reconstruction of images with subdiffraction resolution. Specifically, a biological sample can be labeled with photoactivatable probes in their nonemissive state and illuminated at
with low intensities. Under these conditions, only a small fraction of probes switches to their emissive state (Figure 1.10), ensuring a large physical separation between them. At this point, the activated sample can be illuminated at \( \lambda_{\text{Ex}} \) and the few emissive species can be localized at the single-molecule level with nanoscaled precision, if their brightness and contrast are designed to be sufficiently large. The coordinates of the localized probes can be recorded and the emissive species can be irradiated further at \( \lambda_{\text{Ex}} \) until they bleach. Then, the sequence of activation, excitation, localization and bleaching events can be reiterated multiple times, recording for every single sequence the coordinates of a new subset of probes. Eventually this procedure permits the accumulation of a sufficient number of coordinates to compile a single image of the sample with a lateral resolution that is no longer limited by diffraction. For example, the tubulin structure of PtK2 cells can be immunolabeled with the photochromic rhodamine 4a.\textsuperscript{98} Reiterative sequences of activation, excitation, localization and bleaching events can then be exploited to map the spatial distribution of the fluorescent probes with subdiffraction resolution. Comparison of the resulting image with its diffraction-limited counterpart (\( a \) and \( b \) in Figure 1.10) clearly reveals a drastic enhancement in resolution. Individual tubulin filaments can be distinguished in the former, while they appear as blurred objects in the latter. In fact, this powerful imaging protocol has been extended already to numerous other photoactivatable fluorophores\textsuperscript{57–59,74–76,98,99,103,104,150–157} to permit the visualization of a diversity of biological specimens with a resolution that is otherwise impossible to achieve with conventional probes.
Figure 1.10. The tubulin network of PtK2 cells can be stained with the photoactivatable fluorophore 4a. Illumination at an activating wavelength ($\lambda_{Ac}$) converts 4a into 4b, which can be irradiated at an exciting wavelength ($\lambda_{Ex}$) to emit light in the form of fluorescence and eventually bleach. Reiterative sequences of activation and excitation events can be exploited to localize sequentially multiple fluorescent labels and reconstruct an image (a) with a significant improvement in spatial resolution relative to a conventional diffraction-limited counterpart (b). The scale bar in a corresponds to 2 µm.

1.7. Conclusions

Synthetic fluorophores can be designed to emit light in the form of fluorescence upon excitation at one wavelength only after activation at another wavelength. Such photoactivatable fluorophores can be constructed by connecting a photocleavable group to a fluorescent chromophore. The photoinduced cleavage of the former can be exploited to control the ability of the latter to absorb at the exciting wavelength. Alternatively, this photochemical process can alter the rate of nonradiative decay of the fluorescent component. Both mechanisms translate into irreversible fluorescence activation. Similarly, photochromic transformations can be invoked to control the ability of a fluorescent component to absorb at the exciting wavelength. The inherent reversibility of photochromic processes, however, also offers the opportunity to deactivate the activated fluorophore. Indeed, multiple activation/deactivation cycles can be performed under optical control with these switchable systems.
The photochemical and photophysical behavior of photoactivatable fluorophores permits the control of the spatial and temporal distribution of fluorescence. These unique characteristics translate into the possibility of implementing imaging and spectroscopic protocols that are otherwise impossible with conventional fluorophores. Specifically, the diffusion of chemicals labeled with photoactivatable fluorophores and the dynamics of fluids containing such switchable compounds can both be probed in a diversity of media, relying on the interplay of activation and excitation events. Furthermore, the ability to activate the emission of individual fluorophores at different intervals of time offers the opportunity to temporally resolve probes that are separated by relatively short distances. This protocol can overcome the limitations imposed by diffraction on the resolution of conventional fluorescence images and, hence, enables the visualization of biological specimens at nanometer level. Thus, photoactivatable fluorophores are versatile molecular probes for the noninvasive investigation of the dynamics and structures of a diversity of samples.
CHAPTER 2
SYNTHESIS AND PROPERTIES OF MOLECULAR SWITCHES BASED ON THE OPENING AND CLOSING OF OXAZINE RINGS

2.1. Background

Organic molecules can be designed to undergo pronounced structural changes in response to external stimulation. In turn, these switching events can be exploited to control motion at the molecular level as well as to regulate the electrochemical and spectroscopic signatures of multicomponent assemblies. Indeed, molecular switches are becoming valuable building blocks for the construction of a diversity of functional materials with controllable properties. In particular, photochromic switches offer the opportunity to implement such structural transformations reversibly under the influence of light. The photochemical events associated with these compounds can be accompanied by significant changes in absorption coefficients, dipole moments, fluorescence quantum yields and redox potentials together with pronounced modifications in molecular shapes and dimensions. In fact, the ability to photoregulate these parameters has already translated into the realization of photoresponsive molecular and supramolecular constructs based on photochromic components.

In search of strategies to implement photochromic transformations with fast switching speeds, we developed a family of oxazines able to open and close their heterocyclic core in response to optical stimulation. Specifically, our molecules fuse 2H,3H-indole and 2H,4H-benzo[1,3]oxazine heterocycles in their molecular skeletons. Upon ultraviolet illumination, the [C–O] bond at the junction of the two heterocycles cleaves on a subnanosecond timescale. The photoinduced bond cleavage opens the oxazine ring and generates a phenolate chromophore. As a result, this photochemical process is accompanied by the appearance of an absorption band in the visible region of the electromagnetic spectrum. The photogenerated isomer, however, reverts spontaneously.
to the original one on a submicrosecond timescale. Indeed, these molecules can be switched back and forth between their two interconvertible states hundreds of times with no sign of degradation, even in the presence of molecular oxygen.

In the course of investigating the photochemical and photophysical properties of our photochromic compounds, we discovered that their oxazine ring opens also under the influence of either acid or base with the formation of either a $3H$-indolium or a phenolate chromophore respectively.\textsuperscript{170a,g} As a result, these transformations are, once again, accompanied by significant changes in absorption across the ultraviolet and visible regions of the electromagnetic spectrum. On the basis of these observations, we envisaged the opportunity of exploiting the halochromic and photochromic character of our relatively simple switching subunit to control the properties of an appended component. Specifically, we devised versatile synthetic strategies to attach covalently an oxazine ring to a diversity of chromophoric groups with the ultimate goal of being able to modulate their absorption, electrochemical and/or emission properties under the influence of light, acid and/or base. In order to assess the generality of these operating principles, we designed eight molecular switches all pairing covalently an oxazine ring to an appropriate chromophore. In this chapter, I report the synthesis of these molecules and of appropriate models together with the electrochemical, photochemical and photophysical properties of these functional compounds.

\textbf{2.2. Results and Discussion}

\textbf{2.2.1. Design and Synthesis}

The photoinduced opening (from \textit{a} to \textit{b} in Figure 2.1) of the oxazine ring of our photochromic compounds generates a zwitterionic isomer incorporating a phenolate anion and a $3H$-indolium cation.\textsuperscript{170} This transformation can be exploited to perturb the electronic structure of a chromophoric appendage attached to either position 4 on the
phenoxy fragment (R$^1$ in Figure 2.1) or the chiral center at the junction of the two heterocycles (R$^2$ in Figure 2.1).

![Molecular Switches Diagram](image)

**Figure 2.1.** The molecular switches 9a–15a adopt preferentially a ring-closed form (a) in solution, while 8a is exclusively in the ring-open state (b) under the same conditions. The oxazines 9a, 10a and 12a switch reversibly to the corresponding zwitterionic isomers 9b, 10b and 15b upon ultraviolet illumination. However, 11a and 13a–15a do not undergo this photochemical transformation.

Indeed, the developing charges on the phenolate and 3H-indolium fragments can donate electrons to R$^1$ and withdraw them from R$^2$ respectively. Thus, the photoinduced opening and thermal closing of the oxazine ring can, in principle, be exploited to
modulate the absorption, emission and redox properties of a chromophore attached to either one of these two positions. On the basis of these considerations and the availability of appropriate precursors, we designed the molecular switches 8a–15a (Figure 2.1) and devised synthetic procedures for their preparation in a single step from known starting materials with yields ranging from 16 to 46%. In particular, I condensed the preformed oxazine 16 with the aldehydes 17–22 (Figure 2.2) in the presence of trifluoroacetic acid (TFA) to generate the target switches 8a–13a. Similarly, I condensed the preformed oxazine 23 (Figure 2.3) with the iodide salt of 24 and isolated the hexafluorophosphate salt of the target switch 14a, after counterion exchange. In the case of 15a, instead, I reacted the coumarin 25 (Figure 2.4) with formaldehyde and hydrochloric acid first and then with the 3H-indole 26 to produce the target switch.

![Synthesis of the molecular switches 8a–13a.](image)

**Figure 2.2.** Synthesis of the molecular switches 8a–13a.
In addition to the molecular switches 8a–15a, I also synthesized the model compounds 27–31, 33 and 34 (Figure 2.5). Specifically, I isolated the hexafluorophosphate salts of these 3H-indolium cations in yields ranging from 24 to 85%, after the condensation of the iodide salt of 24 to the corresponding aldehydes and counterion exchange. Compounds 27–31 have essentially the same chromophoric fragment of the ring-open isomer (b in Figure 2.1) of the molecular switches 8a–12a. Similarly, the model 33 and the conjugate base of the phenol 34 resemble the chromophores incorporated within the ring-closed and -open isomers (a and b in Figure 2.1) of 14a.
Figure 2.5. Model compounds 25 and 27–35.

2.2.2 Steady-State Spectroscopy

The steady-state absorption spectrum of 8a (a in Figure 2.6), recorded in acetonitrile at 20 °C, indicates that its ring-open isomer (b in Figure 2.1) is the predominant species in solution. Specifically, the spectrum shows the characteristic absorbance of the 4-nitrophenolate chromophore\textsuperscript{170a} of this isomer at 408 nm. This band shifts to 307 nm (b in Figure 2.6) after the addition of acid and the formation of a 4-nitrophenol chromophore (c in Figure 2.7).\textsuperscript{170g}
Figure 2.6. Steady-state absorption spectra (50 µM, MeCN, 20 °C) of 1 before (a) and after (b) the addition of TFA (5 eq.) and of 27 (c).

![Steady-state absorption spectra](image)

Figure 2.7. Opening of the oxazine ring with the addition of either acid or base

In addition, both spectra show also an intense absorption at ca. 530 nm (λ_{Ab} in Table 2.1.). This band resembles that of the model compound 27 and corresponds to the 3H-indolium chromophore incorporated within both the ring-open isomer (b in Figure 2.1) and its protonated form (c in Figure 2.7). These observations demonstrate that the pyrrole fragment of 8a locks this particular system in the ring-open form. Presumably, the extended conjugation of the 3H-indolium cation, possible only within the ring-open isomer, is mostly responsible for the preferential population of this species over the ring-closed one.
Table 2.1. Photochemical and photophysical parameters [a] of the ring-open isomer of 8a, ring-closed isomers of 9a–15a and their models 25 and 27–34.

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{\text{Ab}} [b] ) (nm)</th>
<th>( \phi_P [c] )</th>
<th>( \tau_P [d] ) (( \mu )s)</th>
<th>( \lambda_{\text{Ab}} [b] ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8b</td>
<td>530</td>
<td>—</td>
<td>—</td>
<td>27</td>
</tr>
<tr>
<td>9a</td>
<td>326</td>
<td>0.03</td>
<td>0.2</td>
<td>28</td>
</tr>
<tr>
<td>10a</td>
<td>288</td>
<td>0.01</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>11a</td>
<td>337</td>
<td>—</td>
<td>—</td>
<td>30</td>
</tr>
<tr>
<td>12a</td>
<td>316</td>
<td>0.01</td>
<td>50</td>
<td>31</td>
</tr>
<tr>
<td>13a</td>
<td>316</td>
<td>—</td>
<td>—</td>
<td>32</td>
</tr>
<tr>
<td>14a</td>
<td>428</td>
<td>—</td>
<td>—</td>
<td>34 [e]</td>
</tr>
<tr>
<td>15a</td>
<td>320</td>
<td>—</td>
<td>—</td>
<td>25 [e]</td>
</tr>
</tbody>
</table>

[a] All parameters were measured in MeCN at 20 °C. [b] The absorption wavelength \( \lambda_{\text{Ab}} \) was estimated from the spectra in Figures 2.6, 2.8, 2.9, 2.10–2.14 [c] The quantum yield \( \phi_P \) for the photochromic transformation was determined with a benzophenone standard, following a literature protocol (ref. 170k). [d] The lifetime \( \tau_P \) of the photogenerated isomer was determined from the temporal absorbance evolutions in Figures 10, S6 and S8. [e] The values of \( \lambda_{\text{Ab}} \) reported for 34 and 25 were measured in the presence of Et\(_3\)N (5 eq.) and Bu\(_4\)NOH (100 eq.) respectively.

In contrast to the behavior of 8a, the spectra of 9a–13a (a in Figures 2.8 and Figures 2.9 – 2.12) indicate that the ring-closed isomer (a in Figure 2.1) is, instead, the predominant species in solution, under otherwise identical conditions. The corresponding spectra reveal bands for the chromophore (\( R^2 \) in Figure 2.1) appended to the chiral center of the oxazine ring at wavelengths ranging from 288 to 337 nm (Table 2.1). Upon addition of acid, the oxazine ring opens and brings \( R^2 \) in conjugation with the adjacent 3\( H \)-indolium cation in the resulting protonated species (c in Figure 2.7).
Figure 2.8. Steady-state absorption spectra (50 μM, MeCN, 20 °C) of 9a before (a) and after (b) the addition of TFA (5 eq.) and of the hexafluorophosphate salt of 28 (c).

Figure 2.9. Steady-state absorption spectra (10 μM, MeCN, 20 °C) of 10a before (a) and after (b) the addition of TFA (10 eq.) and of the hexafluorophosphate salt of 29 (c).

Figure 2.10. Steady-state absorption spectra (10 μM, MeCN, 20 °C) of 11a before (a) and after (b) the addition of TFA (10 eq.) and of the hexafluorophosphate salt of 30 (c).
Figure 2.11. Steady-state absorption spectra (0.1 mM, MeCN, 20 °C) of 12a before (a) and after (b) the addition of TFA (10 eq.) and of the hexafluorophosphate salt of 31 (c).

Figure 2.12. Steady-state absorption spectra (0.1 mM, MeCN, 20 °C) of 13a before (a) and after (b) the addition of TFA (1 eq.) and of 32 (c).

This transformation shifts bathochromically the absorption band of $\text{R}^2$ into the visible region (b in Figures 2.8 and 2.9 – 2.12). In fact, the resulting absorptions resemble those of the model 3H-indolium cations 28–31 (c in Figures 2.8 and 2.9 – 2.11), which are centered at wavelengths ranging from 400 to 529 nm (Table 2.1). Thus, the acid-induced opening of the oxazine rings of 9a–13a alters the electronic structure of their chromophoric appendages and shifts their absorptions into the visible region. In the case of 9a, 10a and 13a, a similar band, albeit significantly less intense, is also observed in the spectra (a in Figures 2.8, 2.9 and 2.12) recorded before the addition of acid. This
absorption indicates the co-existence in solution of a small fraction of ring-open isomer, together with the predominant ring-closed species, for these particular three switches.

The absorption spectra of 14a and 15a (a in Figures 2.13 and 2.14) are also indicative of the predominant presence of the ring-closed isomer in solution. In fact, they reveal bands at 428 and 320 nm respectively (Table 2.1), which resemble those of the model compounds 33 and 35 (c in Figure 2.13 and b in Figure 2.14). Instead, there are no bands in the range of wavelengths where the phenolate chromophores of the ring-open isomers are expected to absorb, according to the spectra of the conjugate bases of the model phenols 34 and 25 (d in Figure 2.13 and c in Figure 2.14). Nonetheless, a weak absorption for the phenolate chromophore can be detected at ca. 390 nm (b in Figure 2.13), after the addition of tetrabutylammonium hydroxide to 15a. Indeed, the oxazine ring of our compounds is known to open under these conditions with the formation of the corresponding hemiaminal (d in Figure 2.7). In the case of 14a, the nucleophilic hydroxide anion attacks also the 3H-indolium cation appended to the phenoxy fragment, in addition to opening the oxazine ring, and prevents the detection of the absorption band for the extended phenolate chromophore.
**Figure 2.13.** Steady-state absorption spectra (50 µM, MeCN, 20 °C) of 15a before (a) and after (b) the addition of Bu₄NOH (100 eq.), of 35 (c) and of 25 (d) after the addition of Bu₄NOH (100 eq.). Steady-state emission spectra (50 µM, MeCN, 20 °C, λₓₑₙ = 405 nm) of 15a before (e) and after (f) the addition of Bu₄NOH (100 eq.), of 35 (g) and of 25 (h) after the addition of Bu₄NOH (100 eq.).

**Figure 2.14.** Steady-state absorption spectra (1 µM, MeCN, 20 °C) of 14a (a), of the hexafluorophosphate salt of 33 (b) and of the hexafluorophosphate salt of 34 (c) after the addition of Et₃N (5 eq.).
The steady-state emission spectra of 8a–13a show negligible fluorescence and do not change significantly after the addition of acid. Similarly, also 14a and 15a (e in Figure 2.13) are essentially not emissive. After the addition of tetrabutylammonium hydroxide, however, a small fraction of the ring-closed isomer of 15a is converted into the corresponding hemiaminal (d in Figure 2.7) and an intense emission band appears at 470 nm (f in Figure 2.13) upon excitation at 405 nm. Indeed, the spectra of the model coumarin 35 (g in Figure 2.13) and of the conjugate base of the model phenol 25 (h in Figure 2.13) reveal that, while the former is virtually not emissive under these excitation conditions, the latter emits with a quantum yield of 0.99. Thus, the base-induced opening of the oxazine ring of 15a alters the electronic structure of the appended coumarin chromophore and switches its fluorescence from off to on.

2.2.3. Time-Resolved Spectroscopy

The steady-state absorption spectra (a in Figures 2.8, 2.13, 2.9 – 2.12 and 2.14) of 9a–15a show that these molecular switches adopt preferentially the ring-closed form (a in Figure 2.1) in acetonitrile at ambient temperature. Upon illumination at 355 nm with a pulsed laser, however, the oxazine ring of 9a, 10a and 12a opens within the duration of the laser pulse (6 ns) to generate the corresponding zwitterionic isomer (b in Figure 2.1) with a quantum yield (ϕP in Table 2.1) ranging from 0.01 to 0.03. Consistently, the absorption spectra (a in Figures 2.15, 2.16 and 2.18), recorded 0.1 µs after excitation, reveal the appearance of bands in the visible region.
Figure 2.15. Time-resolved absorption spectrum (a) of a solution (18 µM, MeCN, 20 °C) of 9a, recorded 0.1 µs after pulsed illumination at 355 nm (15 mJ, 6 ns), and the subsequent absorbance evolution (b) at 550 nm with the corresponding monoexponential fitting.
Figure 2.16. Time-resolved absorption spectrum (a) of a solution (66 µM, MeCN, 20 °C) of 10a, recorded 0.1 µs after pulsed illumination at 355 nm (15 mJ, 6 ns), and the subsequent absorbance evolution (b) at 420 nm with the corresponding monoexponential fitting.
These bands resemble those observed in the steady-state spectra (c in Figures 2.8, 2.9 and 2.11) of the model compounds 28, 29 and 31 and correspond to ground-state absorptions of the 3H-indolium chromophores of the ring-open isomers of 9a, 10a and 12a. The photogenerated isomers, however, revert spontaneously back to the original species and, consistently, their transient absorptions in the visible region decay monoexponentially (b in Figures 2.15, 2.16 and 2.18). Curve fitting of the resulting temporal absorbance profiles indicates the lifetime (\(\tau_p\) in Table 2.1) to range from 0.2 to 50 \(\mu\)s. Interestingly, the lifetimes of the ring-open isomers of 10a and 12a are one order of magnitude longer than that of 9a. Presumably, their thiophene appendages stabilize effectively the 3H-
indolium cation of the ring-open isomer, by extending its conjugation, and delay the ring-closing kinetics.

The time-resolved absorption spectra (a in Figures 2.17 and 2.19) of 11a and 13a, recorded under otherwise identical illumination conditions, also reveal an absorbance increase in the visible region. However, the resulting bands do not resemble those expected for the 3H-indolium cations of the corresponding ring-open isomers (b in Figures 2.10 and 2.12).

![Figure 2.18](image)

**Figure 2.18.** Time-resolved absorption spectrum (a) of a solution (12 µM, MeCN, 20 °C) of 11a, recorded 0.1 µs after pulsed illumination at 355 nm (15 mJ, 6 ns), and the subsequent absorbance evolution at 430 nm under nitrogen (b) and air (c) with the corresponding monoexponential fittings.
The spectrum observed for 11a is, instead, similar to the triplet–triplet absorption of bisthiophene\textsuperscript{172} and its lifetime shortens from 2.7 to 0.3 \(\mu\)s in the presence of molecular oxygen (\(b\) and \(c\) in Figure 2.17). These observations suggest that intersystem crossing follows the excitation of 11a and prevents the opening of its oxazine ring. By contrast, the spectral changes observed upon irradiation of 13a are irreversible. Thus, this particular switch appears to undergo significant degradation under illumination. Presumably, intramolecular electron transfer pathways from the ferrocene appendage to the excited 4-nitrophenoxy chromophore are responsible for the photochemical decomposition of this compound.

In contrast to the behavior of 9a–12a, the transient absorption spectra of 14a and 15a do not reveal any significant change on a nanosecond timescale upon illumination. The inability of their oxazine ring to open upon excitation on this temporal scale is, presumably, a result of the lack of a nitro group in position 4 of their phenoxy fragments. Indeed, all the structural modifications explored at this particular position so far, besides the introduction of either a nitro or a 4-nitrophenyl group, have resulted in the suppression of the photochemical character of the oxazine ring\textsuperscript{170d,l,j}.
2.2.4. Differential Pulse Voltammetry

The steady-state absorption spectrum of 13a (a in Figure 2.12) reveals intense bands for the ring-closed isomer and a weak absorption for the ring-open species. In agreement with the co-existence of both isomers in solution, the differential pulse voltammogram of 13a (a in Figure 2.20) shows two peaks of different intensities at +0.52 and +0.76 V for the oxidation of the ferrocene fragments of the ring-closed and -open species respectively.

Figure 2.20. Differential pulse voltammograms (1.5 mM Bu₄NPF₆, MeCN, 20 °C, V vs. Ag/AgCl, scan rate = 20 mV s⁻¹, pulse amplitude = 50 mV) of 6 before (a) and after (b) the addition of TFA (1 eq.) and of 16 before (c) and after (d) the addition of TFA (20 eq.).

Consistently with this assignment, the opening of the oxazine ring with acid (a and c in Figure 2.7) results in an increase in the intensity of peak at +0.76 V with a concomitant
decrease in that at +0.52 V (b in Figure 2.20). Thus, the electrochemical response of the redox center appended to the switchable unit can, indeed, be controlled by operating the latter with chemical inputs.

In addition to the two peaks for ferrocene oxidation, the differential pulse voltammogram of 13a shows also a third peak at +1.27 V (a in Figure 2.20). This peak resembles that observed for the model oxazine 16 (c in Figure 2.20) and corresponds to the oxidation of the 2H,3H-indole heterocycle. After the addition of acid and the opening of the oxazine ring, this particular fragment becomes positively charged, preventing the oxidation of the heterocyclic fragment, and the peak disappears for both compounds (b and d in Figure 2.20). Thus, the redox response of the switchable unit itself can also be controlled by opening the oxazine ring with chemical stimulation.

2.3. Conclusions

A switchable oxazine can be condensed to a variety of formylated chromophores in a single synthetic step. Either addition of acid or ultraviolet illumination opens the oxazine ring of the resulting constructs and brings the chromophoric fragment in conjugation with a 3H-indolium cation. This pronounced structural transformation extends the conjugation of the chromophore to shift its main absorption from the ultraviolet to the visible region and, in the case of a ferrocene center, also the oxidation potential in the positive direction. Alternatively, chromophoric fragments can be attached to the phenylene ring fused to the oxazine heterocycle. In this instance, the addition of base opens the oxazine ring and brings the chromophoric appendage in conjugation with a phenolate anion. Once again, this pronounced modification in electronic structure alters the absorption properties of the chromophore and, in the case of a coumarin fluorophore, leads also to a significant enhancement in emission intensity. Thus, this particular structural design to switch properties at the molecular level, coupled to the generality of the synthetic strategies,
allows the development of functional materials able to respond to chemical input and optical stimulation.
CHAPTER 3

PHOTOACTIVATABLE FLUOROPHORES FOR SUPER-RESOLUTION IMAGING BASED ON OXAZINE AUXOCHROMES

3.1. Background

Fluorescence imaging\(^2\) offers the opportunity to visualize noninvasively and in real time biological samples labeled with appropriate molecular probes.\(^1\) Indeed, the illumination of a labeled sample at the excitation wavelength of the probes and the detection of their emission with the aid of a confocal microscope permit the reconstruction of three-dimensional images even in living specimens.\(^3\) As a result, the fluorescence microscope has become an indispensable analytical tool in the biomedical laboratory for the convenient investigation of cells and tissues. Illumination and detection, however, require the use of lenses to focus the exciting and emitted radiations respectively.\(^2\) In turn, the phenomenon of diffraction\(^1\) accompanies focusing and imposes a spatial distribution of subwavelength dimensions on the focused radiations. Generally, radiation in the visible region of the electromagnetic spectrum must be employed to visualize biological samples\(^1\) and their relatively long wavelengths translate into spatial resolutions of hundreds of nanometers in the horizontal and vertical directions.\(^3\) These physical dimensions are two orders of magnitude greater than the sizes of most molecules. It follows that conventional fluorescence microscopes cannot provide structural information at the molecular level.

The barrier imposed by diffraction on the spatial resolution of a far-field microscope can be overcome with the aid of switchable fluorophores.\(^25-42\) Specifically, fluorophores co-localized within the same subdiffraction volume can be separated in time, if their fluorescence is designed to switch independently at different intervals of time. In fact, their temporal separation allows the sequential reconstruction of their spatial distribution with nanoscale precision. The implementation of these operating principles, however,
requires probes able to switch from a nonfluorescent state to a fluorescent one, or vice versa, upon illumination at an appropriate wavelength.\textsuperscript{57–59,70,97,103,104,98–101,174} Under these conditions, irradiation of the sample at the switching wavelength with relatively low intensity can be exploited to maintain only a relatively small fraction of probes in the fluorescent state. At this point, illumination of the sample at the excitation wavelength of the fluorescent species permits their localization at the single-molecule level and, eventually, the bleaching of the localized probes. Sequences of switching, localization and bleaching steps can then be reiterated multiple times until a sufficient number of spatial coordinates are available to compile a complete image of the sample with subdiffraction resolution. In alternative to photoinduced switching, binding events in the ground state\textsuperscript{175} and electron transfer processes in the excited state\textsuperscript{176–181} can be invoked to turn fluorescence off, maintain transiently only a portion of probes in an emissive state and permit their localization. Once again, reiterative sequences of switching and localization steps, eventually, permit mapping of the spatial distribution of multiple probes with nanoscale precision and the reconstruction of images.

The photoinduced and reversible interconversion of photochromic compounds\textsuperscript{82–88} can be exploited to switch fluorescence under optical control\textsuperscript{89–94} Indeed, fluorescent and photochromic components can be paired within the same molecular skeleton or supramolecular construct and the emission of the former can be modulated by switching the latter. Generally, the transfer of energy from the excited fluorophore to only one of the two interconvertible states of the photochrome or the exchange of an electron between the excited fluorophore and one of the two states of the photochrome is responsible for fluorescence modulation.\textsuperscript{105–127} On the basis of these mechanisms, we developed several photoswitchable fluorescent systems and operated them in organic and aqueous solutions,\textsuperscript{182} within rigid polymer matrices\textsuperscript{183} and inside living cells.\textsuperscript{184} In the wake of these results, we envisaged the possibility of adapting our structural design to develop photoswitchable fluorophores for super-resolution imaging based on fluorescent
and photochromic components. In particular, we designed a family of fluorophore–photochrome dyads able to switch from a nonfluorescent to a fluorescent state upon illumination. In this article, we report the logic behind their operating principles together with their chemical synthesis and a detailed characterization of their equilibration dynamics, photochemical and photophysical properties.

3.2. Results and Discussion

3.2.1. Design and Synthesis

In search of viable structural designs to construct photochromic compounds with fast switching speeds and excellent fatigue resistances, our group developed a family of molecular switches fusing 2H,3H-indole and benzooxazine heterocycles within a single covalent skeleton (a in Figure 3.1). Upon ultraviolet illumination, the 2H,4H-[1,3]oxazine ring at the core of these compounds opens on a subnanosecond timescale to generate a zwitterionic isomer (b in Figure 3.1). The photogenerated species reverts spontaneously to the original isomer on a submicrosecond timescale. In fact, these compounds can be switched hundreds of times between their two states with no sign of degradation simply by turning on and off an ultraviolet source. Furthermore, we demonstrated that their photochemical transformation can be exploited to control the photophysical properties of an appended aromatic chromophore (R in Figure 3.1). Indeed, the photoinduced ring-opening process brings R in conjugation with the 3H-indolium cation of the photogenerated isomer and alters its electronic structure. As a result, the $S_0 \rightarrow S_1$ absorption of R shifts bathochromically and, in some instances, moves from the ultraviolet up to the visible region of the electromagnetic spectrum. On the basis of these results, I envisaged the possibility of exploiting such photoinduced bathochromic shift in absorption to activate fluorescence. Specifically, I realized that visible illumination can excite R only after photoinduced ring opening with concomitant fluorescence, if this particular chromophoric fragment is chosen to be emissive. Thus, I
devised a general synthetic strategy to append our photoswitchable auxochromic unit to fluorescent fragments and designed the molecular switches 36a–40a (Figure 3.1).185

\[ \text{Figure 3.1. Photoinduced and reversible interconversion of the ring-closed (a) and -open (b) isomers of the molecular switches 36–40 and their transformation into a protonated form (c) upon addition of acid.} \]

I prepared 36a–40a in a single synthetic step from the preformed switch 16 (Figure 3.2) and the corresponding aldehydes 41–45. Specifically, the condensation of 16 with 41–47, in the presence of trifluoroacetic acid (TFA), gave the target molecular switches in yields ranging from 30 to 63%. Following a similar synthetic protocol (Figure 3.3), we also prepared the hexafluorophosphate salts of the model 3H-indolium cations 46–50 (Figure 3.4) in yields ranging from 32 to 82%. These model compounds have virtually the same chromophoric fragments of the zwitterionic isomers 36b–40b (Figure 3.1) and are essential for the spectroscopic characterization of these species.
Figure 3.2. Synthesis of the molecular switches 36a–40a.

Figure 3.3. Synthesis of the hexafluorophosphate salts of 47, 48 and 50.
3.2.2. Nuclear Magnetic Resonance Spectroscopy

The $^1$H nuclear magnetic resonance (NMR) spectra of 36a–39a, recorded in deuterated chloroform at 25 °C, reveal resonances that can be assigned exclusively to the ring-closed isomer. The two sets of diastereotopic methyl protons (Me$^A$ and Me$^B$ in Figure 3.5) of this species, however, resonate as a single and broad peak at 1.3–1.6 ppm. These observations indicate that the two enantiomers of the ring-closed isomer exchange rapidly on the $^1$H NMR timescale, under these experimental conditions. In turn, their interconversion implies that the oxazine ring opens and closes spontaneously with the transient formation of the zwitterionic isomer. In agreement with this interpretation, the analysis of 37a and 39a in deuterated acetonitrile reveals a significant temperature dependence of the resonance associated with Me$^A$ and Me$^B$. For example, the singlet observed for these protons in the case of 39a broadens significantly with a decrease in temperature from 70 to 25 °C (Figure 3.5). Indeed, the rate for the degenerate site
exchange decreases with temperature and slows the interconversion of the two diastereotopic environments, relative to the $^1$H NMR timescale, causing the broadening observed for the resonance of Me$^A$ and Me$^B$. By contrast, the exchange process has negligible influence on the methyl protons (Me$^C$ and Me$^D$) of the diethylamino group on the coumarin appendage and their triplet remains essentially unaffected in shape, despite the temperature change. The assessment of the temperature dependence of the line width, associated with the peak for Me$^A$ and Me$^B$, in the fast-exchange regime$^{187}$ indicates the rate constant ($k_1$ in Table 3.1) for the degenerate site exchange to be $ca. 2.2 \times 10^2$ and $7.0 \times 10^2$ s$^{-1}$ at 25 °C for 37a and 39a respectively. These values correspond to a free-energy of activation ($\Delta G^\ddagger$ in Table 3.1) of 14.3 and 13.6 kcal mol$^{-1}$ respectively with an enthalpy of activation ($\Delta H^\ddagger$ in Table 3.1) of 11.3 and 7.2 kcal mol$^{-1}$ and an entropy of activation ($\Delta S^\ddagger$ in Table 3.1) of $-0.01$ and $-0.02$ kcal mol$^{-1}$ K$^{-1}$ respectively. In summary, these spectroscopic studies demonstrate that the ring-closed isomer is the predominant species in solution for 36a–39a and that its oxazine ring opens and closes to permit the interconversion of its two enantiomers.
Figure 3.5. Partial $^1$H NMR spectra (400 MHz) of 39a (1.3 mM, CD$_3$CN) at various temperatures.

Table 3.1. Kinetic parameters [a] associated with the interconversion of the two enantiomers of 37a and 39a in deuterated acetonitrile at 25°C.

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ (10$^2$ s$^{-1}$)</th>
<th>$\Delta G^\ddagger$ (kcal mol$^{-1}$)</th>
<th>$\Delta H^\ddagger$ (kcal mol$^{-1}$)</th>
<th>$\Delta S^\ddagger$ (kcal mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37a</td>
<td>2.2</td>
<td>14.3</td>
<td>11.3</td>
<td>−0.01</td>
</tr>
<tr>
<td>39a</td>
<td>7.0</td>
<td>13.6</td>
<td>7.2</td>
<td>−0.02</td>
</tr>
</tbody>
</table>

[a] The rate constant ($k_1$) for the degenerate site exchange was measured by $^1$H NMR spectroscopy, probing the temperature dependence of the line width for the resonance of the diastereotopic methyl protons (Me$^A$ and Me$^B$ in Figure 3.5) in the fast exchange regime (ref. 187). The corresponding free-energy of activation ($\Delta G^\ddagger$) and the associated enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) terms were determined from the temperature dependence of $k_1$ (ref. 187).

In contrast to the behavior of 36a–39a, the fifth molecular switch adopts predominantly the ring-open form 40b, under otherwise identical conditions. Indeed, its $^1$H NMR spectrum reveals two sharp singlets at 1.68 and 1.74 ppm, integrating for six protons each, and one more at 3.72 ppm, integrating for three, for its five sets of methyl protons, in place of the broad resonance observed for Me$^A$ and Me$^B$ in the spectra of 36a–39a.
Furthermore, the pair of methylene protons of 40b resonate also as a single sharp peak and their chemical shift is ca. 0.6 ppm greater than that of the corresponding protons 36a–39a. Similar changes in chemical shift are also observed for some of the aromatic protons and are particularly evident for the two protons in the ortho-positions relative to the nitro group. Thus, the diene bridge separating the two heterocyclic fragments of 40b, in conjunction with the ability of one to donate electrons to the other, stabilize this zwitterionic isomer sufficiently to discourage the closing of the oxazine ring.

3.2.3. Steady-State Absorption Spectroscopy

The model compounds 46–49 incorporate essentially the same 3H-indolium chromophores of the ring-opened isomers of 36b–39b and their steady-state absorption spectra (a in Figures 3.6 and 3.7–3.9) show intense bands centered between 412 and 573 nm (λ_{Ab} in Table 3.2) in acetonitrile at 20 °C. These absorptions, however, are not observed in the spectra (b in Figures 3.6 and 3.7–3.9) of the molecular switches, which, instead, reveal bands centered between 288 and 412 nm (λ_{Ab} in Table 3.2) for the chromophoric fragments on the chiral center of their ring-closed isomers 36a–39a.\(^{185}\) On the contrary, the spectra (a and b in 3.10) of 50 and 40b both show virtually the same band at ca. 545 nm (λ_{Ab} in Table 3.2). In addition, the spectrum of 40b also reveals the characteristic absorption at 429 nm of the 4-nitrophenolate anion. These observations confirm that the ring-closed isomers 36a–39a and the ring-open species 40b are the predominant species in solution, in full agreement with the \(^1\)H NMR spectroscopic studies.
Figure 3.6. Steady-state absorption spectra of acetonitrile solutions (2.5 µM, 20 °C) of the hexafluorophosphate salt of 49 (a) and of 39a before (b) and after (d) the addition of TFA (3 eq.). Steady-state absorption spectrum of a methanol solution (2.5 µM, 20 °C) of 39a (c). Steady-state emission spectra of acetonitrile solutions (2.5 µM, 20 °C, λ_{Ex} = 593 nm) of 49 (e) and of 39a before (f) and after (g) the addition of TFA (3 eq.).
Figure 3.7. Steady-state absorption spectra of acetonitrile solutions (10 µM, 20 °C) of the hexafluorophosphate salt of 46 (a) and of 36a before (b) and after (c) the addition of TFA (50 eq.). Steady-state emission spectra of acetonitrile solutions (10 µM, 20 °C, λ_{Ex} = 417 nm) of 46 (d) and of 36a before (e) and after (f) the addition of TFA (50 eq.).
Figure 3.8. Steady-state absorption spectra of acetonitrile solutions (5 μM, 20 °C) of the hexafluorophosphate salt of 47 (a), and of 37a before (b) and after (c) the addition of TFA (10 eq.). Steady-state emission spectra of acetonitrile solutions (10 μM, 20 °C, λ<sub>Ex</sub> = 453 nm) of 47 (d) and of 37a before (e) and after (f) the addition of TFA (10 eq.).
Figure 3.9. Steady-state absorption spectra of acetonitrile solutions (10 µM, 20 °C) of the hexafluorophosphate salt of 48 (a) and of 38a before (b) and after (c) the addition of TFA (10 eq.). Steady-state emission spectra of acetonitrile solutions (10 µM, 20 °C, \( \lambda_{\text{Ex}} = 522 \) nm) of 48 (d) and of 38a before (e) and after (f) the addition of TFA (10 eq.).

Table 3.2. Photochemical and photophysical parameters [a] of the ring-closed isomers 36a–39a, the ring-open species 40b and their models 46–50 in acetonitrile at 20 °C.

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{\text{Ab}} ) [b] (nm)</th>
<th>( \phi_P ) [c]</th>
<th>( \tau_P ) [d] (µs)</th>
<th>( \lambda_{\text{Ab}} ) [b] (nm)</th>
<th>( \lambda_{\text{Em}} ) [b] (nm)</th>
<th>( \phi_F ) [e]</th>
</tr>
</thead>
<tbody>
<tr>
<td>36a</td>
<td>288</td>
<td>0.08</td>
<td>0.1</td>
<td>46</td>
<td>412</td>
<td>542</td>
</tr>
<tr>
<td>37a</td>
<td>299</td>
<td>0.02</td>
<td>0.07</td>
<td>47</td>
<td>431</td>
<td>559</td>
</tr>
<tr>
<td>38a</td>
<td>362</td>
<td>—</td>
<td>—</td>
<td>48</td>
<td>501</td>
<td>630</td>
</tr>
<tr>
<td>39a</td>
<td>412</td>
<td>0.02</td>
<td>0.2</td>
<td>49</td>
<td>573</td>
<td>645</td>
</tr>
<tr>
<td>40b</td>
<td>548</td>
<td>—</td>
<td>—</td>
<td>50</td>
<td>543</td>
<td>565</td>
</tr>
</tbody>
</table>

[a] Part of the data for 36a and 39a as well as their models 46 and 49 were reported previously (ref. 170f,g,n). [b] The absorption (\( \lambda_{\text{Ab}} \)) and emission (\( \lambda_{\text{Em}} \)) wavelengths were estimated from the spectra in Figures 5 and S3–S6. [c] The quantum yield (\( \phi_P \)) for the photochromic transformation was determined with a benzophenone standard, following a literature protocol (ref.170k). [d] The lifetime (\( \tau_P \)) of the photogenerated isomer was determined from the temporal absorbance evolutions in Figures 3.11, 3.12 and 3.13. [e] The fluorescence quantum yield (\( \phi_F \)) was determined with fluorescein and rhodamine B standards, following a literature protocol (ref. 1).
Figure 3.10. Steady-state absorption spectra of acetonitrile solutions (10 μM, 20 °C) of the hexafluorophosphate salt of 50 (a) and 40b before (b) and after (c) the addition of TFA (1 eq.). Steady-state emission spectra of acetonitrile solutions (10 μM, 20 °C, λ_{Ex} = 475 nm) of 50 (d) and of 40b before (e) and after (f) the addition of TFA (1 eq.).

The ring-open isomers 36b–39b have zwitterionic character and, as a result, are more polar than the ring-closed species 36a–34a. It follows that the equilibrium between the two isomers is sensitive to solvent polarity and shifts partially toward the zwitterionic species with a transition from acetonitrile to methanol. This effect is particularly evident in the case of 39a. Indeed, the corresponding steady-state absorption spectrum (c in Figure 3.6), recorded in methanol at 20 °C, shows the characteristic absorption of the 3H-indolium chromophore, associated with the ring-open isomer, at 586 nm. The absorbance of this band indicates the ratio between ring-closed and -open isomers to be 93:7 at equilibrium.
In alternative to a change in solvent polarity, the addition of acid can also be exploited to detect the characteristic absorption of the 3H-indolium chromophore in the steady-state absorption spectrum. Indeed, the protonation of the 4-nitrophenolate anion of the zwitterionic species prevents ring closing and converts quantitatively the two equilibrating isomers into the corresponding protonated form (c in Figure 3.1). Consistently, the spectra (d in Figure 3.6 and c in Figures 3.7–3.9), recorded in acetonitrile at 20 °C after the addition of an appropriate number of equivalents of TFA to 36a–39a, show the intense bands of the 3H-indolium chromophores of 36c–39c in the visible region. Instead, a similar treatment of 40b has essentially no influence on the band at 548 nm for its 3H-indolium cation (c in Figure 3.10), but results in the disappearance of the band at 429 nm for its 4-nitrophenolate anion, confirming the assignment of both absorptions and, once again, the fact that this species adopts predominantly the ring-open form.

3.2.4. Steady-State Emission Spectroscopy

The steady-state emission spectra (e in Figure 3.6 and d in Figures 3.7–3.9) of the hexafluorophosphate salts of 46–50 reveal bands centered between 542 and 645 nm (λ_{Em} in Table 3.2) in acetonitrile at 20 °C. The fluorescence quantum yields (φ_{F} in Table 3.2) of 46–48, however, barely approach 0.01, while those of 49 and 50 are 0.09 and 0.03 respectively. Similar bands cannot be detected in the spectra (f in Figure 3.6 and e in Figures 3.7–3.9) of 36a–39a, recorded under otherwise identical conditions. Instead, an emission centered at 564 nm is evident in the spectrum of 40b (e in Figure 3.10). These observations confirm, once again, that the ring-closed isomers 36a–39a and the ring-open form 40b are predominant species in solution. Nonetheless, the addition of acid to acetonitrile solutions of 36a–39a converts them quantitatively to the corresponding protonated forms 36c–39c with the concomitant appearance of the characteristic emission of their 3H-indolium chromophores (g in Figure 3.6 and f in Figures 3.7–3.9). Interestingly, the addition of acid to 40b leads to an enhancement in emission intensity (f
in Figures 3.10), suggesting that the protonation the 4-nitrophenolate anion facilitates the radiative deactivation of the adjacent 3H-indolium cation.

### 3.2.5. Time-Resolved Absorption Spectroscopy

The illumination of acetonitrile solutions of 36a, 37a and 39a with a pulsed laser, operating at 355 nm, excites the 4-nitrophenoxo chromophore and cleaves the [C–O] bond connecting this fragment to the chiral center.\(^{185}\) This process generates the ring-open isomers 36b, 37b and 39b with quantum yields (\(\phi_P\) in Table 3.2) of 0.08, 0.02 and 0.02 respectively. Consistently, the absorption spectra (\(a\) in Figures 3.11, 3.12 and 3.13), recorded after excitation, show the appearance of bands centered at 430, 420 and 570 nm respectively, corresponding to the sum of the ground-state absorptions of the 3H-indolium and 4-nitrophenolate chromophores of 36b, 37b and 39b. The photogenerated species revert spontaneously back to the ring-closed ones with first-order kinetics and a concomitant absorbance decay (\(b\) in Figures 3.11, 3.12 and 3.13) in the visible region. Monoexponential fitting of the temporal absorbance evolution reveals the lifetimes (\(\tau_P\) in Table 3.2) of 36b, 37b and 39b to be 0.1, 0.07 and 0.2 \(\mu\)s respectively in acetonitrile at 20 \(^\circ\)C.\(^{189}\)
Figure 3.11. Time-resolved absorption spectrum (a) of an acetonitrile solution (10 μM, 20 °C) of 39a, recorded 0.03 μs after pulsed illumination at 355 nm (10 mJ, 6 ns), and the subsequent absorbance evolution (b) at 580 nm with the corresponding monoexponential fitting. Time-resolved emission spectra of an acetonitrile solution (10 μM, 20 °C) of 39a, recorded upon pulsed illumination exclusively at 355 nm (10 mJ, 6 ns) (c) or simultaneously at 355 nm (10 mJ, 6 ns) and 532 nm (30 mJ, 6 ns) (d).
Figure 3.12. Time-resolved absorption spectrum (a) of an acetonitrile solution (30 µM, 20 °C) of 36a, recorded 0.08 µs after pulsed illumination at 355 nm (10 mJ, 6 ns), and the subsequent absorbance evolution (b) at 430 nm with the corresponding monoexponential fitting. Time-resolved emission spectrum (c) of an acetonitrile solution (30 µM, 20 °C) of 36a, recorded upon pulsed illumination at 355 nm (10 mJ, 6 ns).
Figure 3.13. Time-resolved absorption spectrum (a) of an acetonitrile solution (0.1 mM, 20 °C) of 37a, recorded 0.1 μs after pulsed illumination at 355 nm (10 mJ, 6 ns), and the subsequent absorbance evolution (b) at 420 nm with the corresponding monoexponential fitting. Time-resolved emission spectrum (c) of an acetonitrile solution (0.1 mM, 20 °C) of 37a, recorded upon pulsed illumination at 355 nm (10 mJ, 6 ns).

The illumination of an acetonitrile solution of 38a, under otherwise identical conditions, also results in a transient absorption in the visible region (a in Figure 3.14), which decays on a microsecond timescale (b in Figure 3.14). However, this band is positioned at 440 nm, while the ground-state absorption associated with the 3H-indolium chromophore of the ring-open isomer 38b should be centered around 500 nm, according to the steady-state spectrum (a in Figure 3.9) of the model compound 48. Furthermore, the lifetime of this transient is 4 μs and this value is one order of magnitude longer than those observed for 36b, 37b and 39b. In fact, the transient band observed upon excitation of 38a can be
assigned to an absorption in the triplet manifold of its pyrene fragment. Indeed, the pyrene chromophore can absorb a significant fraction of the exciting photons at 355 nm and its excitation, eventually, leads to intersystem crossing, rather than to the opening of the adjacent oxazine ring.

![Absorption Spectrum](image)

**Figure 3.14.** Time-resolved absorption spectrum (a) of an acetonitrile solution (10 μM, 20 °C) of 38a, recorded 0.1 μs after pulsed illumination at 355 nm (10 mJ, 6 ns), and the subsequent absorbance evolution (b) at 440 nm

### 3.2.6. Time-Resolved Emission Spectroscopy

The steady-state emission spectra of acetonitrile solutions of 36a, 37a and 39a (f in Figures 3.6 and e in Figures 3.7 and 3.8) do not reveal any significant fluorescence upon illumination in the region of wavelengths where their ring-open isomers absorb. In fact,
the concentrations of $36b$, $37b$ and $39b$ at equilibrium are negligible and, therefore, their fluorescence cannot be detected in ensemble measurements. The photoinduced transformation of the ring-closed isomers into the ring-opened ones, however, can be exploited to transiently populate the fluorescent species and permit detection of their fluorescence. Specifically, illumination of $36a$, $37a$ and $39a$ with a pulsed laser, operating at 355 nm, opens their oxazine ring within the duration of the pulse (6 ns). The $3H$-indolium chromophores of the resulting ring-open isomers $36b$, $37b$ and $39b$ absorb at 355 nm with molar extinction coefficients of 9.9, 4.8 and 5.9 mM$^{-1}$ cm$^{-1}$ respectively, according to the steady-state spectra of the model compounds $46$, $47$ and $49$ ($a$ in Figures 3.6, 3.7 and 3.8). As a result, the sequential absorption of two-photons within the very same laser pulse can open the oxazine ring of one isomer and excite the fluorescent chromophores of the other. Consistently, the emission spectra ($c$ in Figure 3.11, 3.12 and 3.13), recorded under these conditions, reveal bands that resemble the steady-state emissions of the model compounds $46$, $47$ and $49$ ($e$ in Figure 3.6 and $d$ in Figures 3.7 and 3.8).

The steady-state absorption spectra of the model compounds $46$, $47$ and $49$ ($a$ in Figures 3.6, 3.7 and 3.8) indicate that their visible bands are centered at 412, 431 and 573 nm ($\lambda_{Ab}$ in Table 3.1) respectively with molar extinction coefficients of 35, 19 and 83 mM$^{-1}$ cm$^{-1}$ respectively. Thus, the $3H$-indolium chromophores of the ring-open isomers $36b$, $37b$ and $39b$ can absorb more photons at wavelengths comprised within these bands than at 355 nm. As a result, simultaneous illumination at 355 nm, to open the oxazine ring, and at a visible wavelength appropriate to excite the ring-open isomer, translates into a significant increase in the detected emission intensity. In particular, the emission spectrum ($d$ in Figure 3.11), recorded upon pulsed illumination of $39a$ at 355 and 532 nm, shows an emission band centered at 650 nm with an enhancement in intensity of approximately one order of magnitude, relative to that detected upon irradiation at 355 nm only. Thus, these experiments confirm that the ring-open isomers are fluorescent and
demonstrate that the emission of these molecular switches can be activated photochemically.

**3.2.7. Single-Molecule Imaging and Spectroscopy**

Encouraged by the results of the ensemble spectroscopic measurements, I decided to investigate the emissive behavior of our compounds at the single-molecule level. I selected the molecular switch 39a and its model 49 for these studies, because the corresponding 3H-indolium chromophore has the greatest quantum yield ($\phi_F$ in Table 3.2) out of the five systems. I first incorporated both compounds in polymer matrices, by spin coating solutions of each dye and either poly(vinyl alcohol) (PVA) or poly(methylmethacrylate) (PMMA) in an appropriate co-solvent on glass slides, and then investigated their spectroscopic response. I found a distinct behavior for 39a in the two polymers used. In PVA, a large fraction of molecules are already in an emissive state. Moreover, they display blinking dynamics with on- and off-times on the same order of magnitude of those observed for the model compound 49, under similar experimental conditions. Therefore, PVA provides a sufficiently-polar environment to encourage the formation of a significant amount of the ring-open isomer 49b, as observed in methanol solution (c in Figure 3.6). Furthermore, the fluorescence blinking, displayed by 39b and 49 under these conditions, can be assigned to the incursion of triplet and other dark states with similar characteristics for both compounds. By contrast, PMMA films doped with 39a show very few fluorescent molecules and their behavior is clearly different from that of analogous films containing 49. Nonetheless, 39a can be switched within the PMMA matrix to a fluorescent state with either chemical or optical stimulation. In the first instance, exposure of the doped PMMA film to fumes of hydrochloric acid encourages the formation of the protonated form 39c (Figure 3.1) with the concomitant appearance of bright spots in the corresponding image (Figure 3.15). In the second instance, illumination of the doped PMMA film at 355 nm results in the formation of the ring-open isomer 39b and, once again, the appearance of bright spots in the corresponding image.
(Figure 3.16). For this experiment, the activating laser was focused in a portion of the observed area (circled in D of Figure 3.16), while excitation at 532 nm was performed in a wide-field mode, thus covering a region much larger than the imaged one. A clear difference is observed in the image between the areas with and without ultraviolet irradiation; the bright spots appear almost exclusively in the irradiated one. These results suggest that the fluorescent switch 39a, in nonpolar polymeric environments, is an excellent candidate for super-resolution imaging. Indeed, (1) most molecules are initially in a dark state, allowing for a dense staining of a target object, (2) photoactivation can reliably be achieved with a wavelength available to standard microscopes and (3) the brightness and fatigue resistance are sufficient to detect and localize the probes, during incursions of the emissive isomer 39b. Moreover, the activation and excitation intensities and the frame times, employed for the experiments illustrated in 3.16, are of the same order of magnitude of those routinely used for the acquisition of super-resolution images.$^{98,157}$

![Figure 3.15](image)

**Figure 3.15.** Single-molecule images (frame time = 30 ms) of a PMMA film doped with 39a (1 × 10^{-5}% w/w), recorded upon illumination with a continuous-wave laser operating at 532 nm (5 kW cm^{-2}) before and after exposure to HCl_{conc.} fumes for 5 min to encourage the protonation of the dopant (39a + H^+ → 39c). The amount of fluorescent species that can be localized, averaged in different areas of 30 × 30 µm^2, increases from 8 to 97 after protonation. Control images with compound 49 showed no significant difference after exposure to acid.
Figure 3.16. Single-molecule images (frame time = 10 ms) of a PMMA film doped with 39a ($1 \times 10^{-5}$% w/w). Excitation (532 nm, 5 kW cm$^{-2}$) was performed in the wide-field mode, covering the entire image, while activation (355 nm, 10 W cm$^{-2}$) was focused in an area with a radius of ca. 6 µm (circled in D). Three typical frames are shown in A–C and the maximum intensity projection from the whole series of 2,000 frames is displayed in D (the activation laser was turned on at frame 400). Frame A was recorded before turning on the activating laser, while B and C were recorded after activation. In D, the area irradiated at 355 nm, where most activation events ($39a + h\nu \rightarrow 39b$) are photoinduced, is clearly evident. Two single-molecule trajectories are shown in E.

3.2.8. Super-Resolution Imaging

In order to assess the potential of our operating principles in the reconstruction of super-resolution images, we labeled test objects of nanoscale dimensions with compound 39a. Indeed, we recently demonstrated that the amphiphilic co-polymer 51 (Figure 3.17) forms nanoparticles in aqueous environments capable of encapsulating hydrophobic chromophores in their interior.$^{184}$ This particular macromolecule has hydrophilic poly(ethylene glycol) chains and hydrophobic decyl arms appended to a common poly(methacrylate) backbone that provides a relatively nonpolar environment analogous to that of a PMMA matrix. Following a similar experimental protocol, I doped nanoparticles of 51 with the molecular switch 39a. In brief, I dissolved 39a and 51 in
chloroform, flushed the solution with nitrogen to encourage the evaporation of the solvent and re-dissolved the residue in neutral (pH = 7.0) phosphate saline buffer (PBS). After vigorous shaking, I filtered the dispersion, deposited an aliquot (ca. 20 µL) of the filtrate on a glass slide and, after drying in air, imaged the residue. The corresponding transmission electron microscopy (TEM) image (Figure 3.18) clearly shows the presence of nanostructured objects with an average size of 14.2 ± 1.5 nm.

Figure 3.17. Distribution of detected photons per single-molecule event extracted from the data in Figure 3.19. A geometrical-distribution fit yielded an expectation value of 222 photons.

Figure 3.18. TEM image of nanoparticles of 51 doped with 39a. The large square-shaped objects (left) are presumably microcrystals of the inorganic salts present in the buffer solution. The average size of the polymer nanoparticles is 14.2 ± 1.5 nm.
The sample was imaged also with a custom-built wide-field epi-fluorescence microscope, equipped with a fast electron multiplying charge-coupled device (EMCCD) camera for detection and with two laser sources operating at 532 nm and 355 nm for excitation and activation respectively. In order to minimize background and optimize localization, the frame-rate of the camera was adjusted to 10 ms. Typically, sets of 50,000–90,000 frames were collected, and localization of single-molecule events and image reconstruction were performed as previously described. The intensity of the activation laser was set to a low value (typically < 1W cm$^{-2}$) initially and then it was slightly increased during the measurement (up to ca. 100 W cm$^{-2}$) to maintain a sparse distribution of molecules in the bright state. Figure 3.19 shows a super-resolution image (B) of the nanoparticles, reconstructed by localizing single molecules on the basis of the photoinduced interconversion of the nonemissive isomer 39a into the emissive one 39b (Figure 3.1). The pronounced difference between the conventional (wide-field) image (A in Figure 3.19) and the super-resolution counterpart (B) is even more evident in the enlarged areas (C and D). A profile of the emission intensity detected along the two brightest nanoparticles of Figure 3.19, with a peak-to-peak separation of 110 nm, is shown in Figure 3.20, emphasizing the resolving power of the method. The average full width at half maximum (FWHM) for the nanoparticles is ca. 30 nm, in agreement with the average localization precision calculated from the data of Figure 3.20. The latter is given by $\Delta r \approx s / (<n> / 2)^{1/2}$, where $s$ is the FWHM of the diffraction-limited spot on the camera and $<n>$ is the average number of photons detected per recorded event. The reduction of the localization precision by a factor of two is a result of the increase in noise introduced by the EMCCD camera at high-gain factors. In our experiment, $<n>$ is equal to 222 (Figure 3.17 ) and $s$ was estimated to be 300 nm, yielding a localization precision of ca. 28 nm.
Figure 3.19. Fluorescence images of nanoparticles of 51 doped with 39a reconstructed from a series of 90,000 frames (frame time = 10 ms). (A) Wide-field image corresponding to the sum of the whole series, after background subtraction (pixel size = 130 nm). (B) Super-resolution image with 4,493 localized events (pixel size = 20 nm). Two areas are enlarged (left, wide-field; right, super-resolution) in C (688 localized events) and D (431 localized events). The dark spots in A (and C) are probably microcrystals of the inorganic salts present in the buffer solution (see Figure 3.18).

Figure 3.20. (A) Profile of the emission intensity measured along the two brightest nanoparticles in the super-resolution image D of Figure 3.20. The peak-to-peak separation is ca. 110 nm and the average FWHM for the nanoparticles is ca. 30 nm. (B) The time trace of the signal recorded from an area of 5 × 5 pixels, extracted from the frame series of Figure 8 (frame time = 10 ms), showing single-molecule bursts. Because of the size of the area and the stochastic nature of the switching process, each event may correspond to different molecules encapsulated within the same nanoparticle or in different ones.
3.3. Conclusions

Formylated fluorophores can be condensed to a preformed oxazine in a single step under acidic conditions. In acetonitrile at ambient temperature, four of the five resulting molecular constructs retain the oxazine ring in the closed form. However, three of these four compounds ring open upon ultraviolet illumination in less than 6 ns. The photoinduced process brings their fluorescent appendage in conjugation with the 3H-indolium cation of the photogenerated species and shifts its S₀ → S₁ absorption from the ultraviolet to the visible region. As a result, the concomitant visible illumination of the sample can excite the fluorescent fragment selectively within the ring-open isomer. It follows that the irradiation of the sample with an ultraviolet source to open the oxazine ring together with a visible one to excite the photogenerated isomer results in significant fluorescence. Furthermore, the ring-open isomer is sufficiently bright to be detected at the single-molecule level with signal-to-noise ratios appropriate for localization with nanoscale precision. In fact, this particular design to photoactivate the fluorescence of individual molecules offers the opportunity to reconstruct images of polymer nanoparticles with subdiffraction resolution. Thus, our fluorescent molecular switches can eventually evolve into valuable probes for the convenient super-resolution imaging of biological samples.
CHAPTER 4

INSIGHTS ON THE ISOMERIZATION OF PHOTOCHROMIC OXAZINES FROM THE EXCITATION DYNAMICS OF BODIPY-OXAZINE DYADS

4.1. Background

Photochromic compounds\textsuperscript{82–87} undergo reversible photochemical transformations with concomitant changes in their stereoelectronic signature. As a result, these processes can be exploited to modulate properties at the molecular level under optical control. In particular, the dimensions and shape of a photochromic molecule, its dipole moment, redox potentials and spectroscopic response can all be engineered to vary significantly with the photochemical transformation. In turn, the ability to photoregulate these parameters offers the opportunity to design and construct functional molecular assemblies with specific properties. Indeed, photochromic components have already been integrated and operated successfully within a diversity of photoresponsive constructs.\textsuperscript{164–169} The design and investigation of these ingenious molecular switches\textsuperscript{162} is mostly motivated by the need to understand the fundamental factors that regulate the excitation dynamics of multichromophoric assemblies. In addition, these chemical systems might eventually evolve into valuable functional materials for a variety of photonic applications. For example, photochromic compounds can be paired with fluorescent partners in the same molecular skeleton and the emission of the latter can be switched reversibly by operating the former with light.\textsuperscript{87,164–169} Generally, intercomponent electron and energy transfer processes are the dominant mechanisms responsible for fluorescence modulation in these systems.\textsuperscript{105–127} Specifically, one of the interconvertible states of the photochromic component can be designed to accept/donate an electron or accept energy from the excited fluorescent component and quench its emission. Under these conditions, the reversible interconversion of the photochromic element results in fluorescence modulation and the possibility to photoswitch emission permits the optical manipulation
of information at the molecular level\textsuperscript{87,164–169,192} as well as the reconstruction of fluorescence images with nanoscaled resolution.\textsuperscript{25–42}

Recently, our group developed a family of photochromic compounds based on the photoinduced opening and thermal closing of oxazine rings.\textsuperscript{170} In particular, the central core of my molecules fuses 2\textit{H},3\textit{H}-indole and 2\textit{H},4\textit{H}-benzo[1,3]oxazine fragments. Upon ultraviolet illumination, the [C–O] bond at the junction of the two heterocycles cleaves on a subnanosecond timescale to generate a zwitterionic isomer with a phenolate chromophore. As a result, this photochemical transformation is accompanied by the appearance of an absorption band in the visible region of the electromagnetic spectrum. This transient absorption decays monoexponentially with the spontaneous reformation of the [C–O] bond and the regeneration of the original colorless isomer. The substituents around the basic heterocyclic core of these compounds can be manipulated to tune the position of the absorption band of the photogenerated isomer across the visible region as well as to regulate its lifetime from tens of nanoseconds to few microseconds. Furthermore, these molecules have excellent fatigue resistances and withstand hundreds of switching cycles with no sign of degradation, even when they are operated in the presence of molecular oxygen. Despite significant synthetic efforts and extensive spectroscopic analyses, however, the excitation dynamics of this class of photochromic compounds remain elusive. Specifically, it is not clear if the photoinduced opening of their oxazine ring occurs in the singlet manifold or along the potential energy surface of a triplet state. The understanding of such mechanistic implications on the photochemistry of this family of photochromes is of paramount importance in guiding the structural design of novel members with improved properties and their integration within functional multichromophoric assemblies.

Intrigued by the unique combination of photochemical and photophysical properties associated with my photochromic oxazines, our group envisaged the possibility of pairing them covalently to appropriate fluorescent partners with the ultimate goal of developing
fast and stable fluorescent switches. In the wake of these results, I designed five fluorophore–photochrome dyads, each incorporating a borondipyrromethene (BODIPY) fluorophore and an oxazine photochrome. Rather than the anticipated strategy for fluorescence switching, however, this structural design translated unexpectedly into a valuable method to probe the excitation dynamics of the oxazine component and provided, finally, conclusive evidence on the mechanism responsible for the photoinduced ring opening of this family of photochromic compounds. In this article, I report the synthesis of these dyads and appropriate model compounds together with a detailed spectroscopic investigation of their photochemical and photophysical properties.

4.2. Results and Discussion

4.2.1. Design and Synthesis

I designed five BODIPY–oxazine dyads (52a–56a in Figure 4.1) differing in the mode of attachment of their two functional fragments. In compound 52a, the BODIPY fluorophore is connected to position 4 of the phenoxy ring associated with the oxazine photochrome. In compounds 52a–56a, the fluorophore is attached to the chiral center at the junction of the two heterocycles of the photochrome through a 1,4-phenylene, 1,4-phenylene-1,2-ethenediy, 1,2-ethenediy or 4-catechol-1,2-ethenediy spacer respectively. In compounds 52a–55a, one of the carbon atoms along the conjugated platform of the fluorophore is connected to the photochrome either directly or through a spacer. In compound 56a, the boron center of the fluorophore is linked to the catechol ligand appended to the photochrome.
Figure 4.1. Expected photoinduced and reversible interconversion of the ring-closed (a) and -open (b) isomers of the molecular switches 52–56.

I prepared the BODIPY–oxazine dyad 52a in two steps (Figure 4.2), starting from the known precursors\textsuperscript{170a,193} 57 and 26 in an overall yield of 10%. Specifically, I first condensed 57 and 26 to generate the oxazine 58. Then, I reacted this species with 3-ethyl-2,4-dimethylpyrrole, in the presence of trifluoroacetic acid (TFA) and under the assistance of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and boron trifluoride, to
produce the target molecule 52a. Following a similar synthetic strategy, I also prepared the dyad 53a in four synthetic steps (Figure 4.3), starting from commercial reactants with an overall yield of 17%. In particular, I condensed \(i\)-propyltolylketone and phenylhydrazine, in the presence of acetic acid, to generate 59. Then, I brominated the tolyl residue of this species with \(N\)-bromosuccinimide (NBS) and azobisisobutyronitrile (AIBN) and oxidized the resulting intermediate with silver nitrate in water to produce the aldehyde 60. Finally, I condensed 60 with 2-chloromethyl-4-nitrophenol to generate the oxazine 61 and coupled this species with 3-ethyl-2,4-dimethylpyrrole, in the presence of TFA and then under the assistance of tetracyano-1,4-benzoquinone (TCBQ), boron trifluoride and triethylamine, to generate the target dyad 53a.

**Figure 4.2.** Synthesis of the dyad 52a.
Figure 4.3. Synthesis of the dyads 53a and 54a.

I relied on the preformed oxazine 61 also for the preparation of the dyad 54a (Figure 4.3). Specifically, I condensed 61 with the known \textsuperscript{194} BODIPY 62, in the presence of acetic acid and piperidine, to produce 54a in a yield of 19%. Following a similar protocol, I also condensed the known oxazine \textsuperscript{170a,195} 16 (Figure 4.4) with the BODIPY 63 to
generate 55a in a yield of 10%. Alternatively, I reacted 2,3,3-trimethyl-3H-indole with 3,4-dihydroxybenzaldehyde, in the presence of hydrobromic acid, to produce 64 (Figure 4.5) and then chelated the boron center of 62 with the catechol appendage of 64, under the assistance of aluminum chloride, to generate 65. Finally, I reacted 65 with 2-chloromethyl-4-nitrophenol to produce the dyad 56a in an overall yield of 13%.

**Figure 4.4.** Synthesis of the dyad 55a.
Figure 4.5. Synthesis of the dyad 56a.

In addition to synthesizing 52a–56a, I also prepared the known molecules 62, 66, 68 and 70 and new compounds 67, 69 and 71–74 (Figure 4.6) to facilitate the spectroscopic characterization of the BODIPY–oxazine dyads. In fact, 62 and 66–69 are equivalent to the BODIPY fluorophores incorporated within the ring-closed isomers 52a–56a, while the conjugate base of 70 resembles the phenolate chromophore of the ring-open isomer 52b. Similarly, 71–74 are equivalent to the 3H-indolium cations of the ring-open isomers 53b–56b. I synthesized 67 and 69 in one step from 62. Similarly, I
prepared 71–74 in one or two steps (Figure 4.7–4.9), adapting the experimental procedures for the preparation of the corresponding dyads.

Figure 4.6. The model compounds 62 and 66–74.
Figure 4.7. Synthesis of 71 and 72.

Figure 4.8. Synthesis of 73.

Figure 4.9. Synthesis of 74.
4.2.2. Steady-State Spectroscopy

The absorption spectrum (\(a\) in Figure 4.10) of the dyad 52a shows a band centered at 521 nm (\(\lambda_{Ab}\) in Table 4.1). This band is essentially identical to that (\(c\) in Figure 4.10) of the model 66 and corresponds to the BODIPY component. Upon addition of base, the oxazine ring of the photochromic component opens (Figure 4.11) to produce the hemiaminal 52c.\(^{170a}\) The absorption spectrum (\(b\) in Figure 4.10), however, does not reveal any significant change in the visible region. These observations indicate that the phenolate and BODIPY fragments of the resulting species 52c are not in conjugation. In agreement with the lack of any electronic communication in the ground state between these two subunits, also the absorption spectrum (\(d\) in Figure 4.10) of the model phenol 70 does not change after the addition of base and remains essentially identical to that of 66.

**Figure 4.10.** Steady-state absorption spectra (10 \(\mu\)M, MeCN, 20 °C) of 1a before (\(a\)) and after (\(b\)) the addition of Bu4NOH (100 eq.), of 66 (\(c\)) and of 70 (\(d\)) after the addition of Bu4NOH (100 eq.). Steady-state emission spectra (1 \(\mu\)M, MeCN, 20 °C, \(\lambda_{Ex} = 500\) nm) of 52a before (\(e\)) and after (\(f\)) the addition of Bu4NOH (100 eq.), of 66 (\(g\)) and of 70 (\(h\)) after the addition of Bu4NOH (100 eq.).
Figure 4.11. Opening of the oxazine ring (a) upon addition of base (c) or acid (d).

The emission spectrum (e in Figure 4.10) of 52a reveals the characteristic BODIPY fluorescence at 532 nm ($\lambda_{Em}$ in Table 4.1). This band does not change after the addition of base and the formation of 52c (f in Figure 4.10). Similarly, the model 66 and the phenolate of 70 have virtually the same emission spectrum (g and h in Figure 4.10) with a quantum yield of ca. 0.7 ($\phi_F$ in Table 4.1). Thus, the opening of the oxazine ring of 52a has a negligible influence on the spectroscopic response of its BODIPY component.

Table 4.1 Photophysical parameters of the dyads and their model compounds in MeCN at 20 °C.

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<th>$\lambda_{Ab}$ [a] (nm)</th>
<th>$\lambda_{Em}$ [a] (nm)</th>
<th>$\tau$ [b] (µs)</th>
<th>$\lambda_{Ab}$ [a] (nm)</th>
<th>$\lambda_{Em}$ [a] (nm)</th>
<th>$\phi_F$ [c]</th>
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[a] The absorption ($\lambda_{Ab}$) and emission ($\lambda_{Em}$) wavelengths were estimated from the spectra in Figure 4.10, 4.12–4.14. [b] The triplet lifetime ($\tau$) was estimated from the monoexponential fitting of the temporal absorbance profiles in Figure 4.15 and 4.17. [c] The fluorescence quantum yield ($\phi_F$) was determined with fluorescein and rhodamine B standards, following a literature protocol (ref. 1). [d] The parameters of 69 were measured after the addition of Bu₄NOH (100 eq.).

The absorption spectra (a in Figure 4.12 and 4.13) of 53a and 54a show bands for their BODIPY components centered at 522 and 573 nm respectively. These absorptions are essentially identical to those (c in Figure 4.12 and 4.13) of the corresponding models 62 and 67 respectively. Upon addition of acid, the oxazine ring of the photochromic component of 53a and 54a opens to generate the corresponding cations 53d and 54d.
respectively (Figure 4.11). The absorption spectra (b in Figure 4.12 and 4.13), however, remain essentially unaffected, indicating the lack of any significant electronic communication between the 3H-indolium and BODIPY fragments in the ground state. Consistently, the absorption bands (d in Figure 4.12 and 4.13) of the model cations 71 and 72 are also virtually identical to those of 62 and 67 respectively. Presumably, steric hindrance prevents the 3H-indolium, 1,4-phenylene and BODIPY subunits from adopting the coplanar geometry necessary to extend conjugation.

![Figure 4.12](image.png)

**Figure 4.12.** Steady-state absorption spectra (1 μM, MeCN, 20 °C) of 53a before (a) and after (b) the addition of TFA (2000 eq.), of 62 (c) and of 71 (d). Steady-state emission spectra (1 μM, MeCN, 20 °C, λ<sub>E</sub> = 480 nm) of 53a before (e) and after (f) the addition of TFA (2000 eq.), of 62 (g) and of 71 (h).
Figure 4.13. Steady-state absorption spectra (5 µM, MeCN, 20 °C) of 54a before (a) and after (b) the addition of TFA (2000 eq.), of 67 (c) and of 72 (d). Steady-state emission spectra (5 µM, MeCN, 20 °C, $\lambda_{\text{Ex}} = 500$ nm) of 54a before (e) and after (f) the addition of TFA (2000 eq.), of 67 (g) and of 72 (h).

The emission spectra (e in Figure 4.12 and 4.13) of 53a and 54a reveal the characteristic fluorescence of their BODIPY components at 535 and 588 nm respectively. These bands are equivalent to those (g in Figure 4.12 and 4.13) associated with the models 62 and 67 respectively. Upon addition of acid to 53a and 54a, however, the emission intensity decreases significantly with the formation of 53d and 54d respectively (f in Figure 4.12 and 4.13). Similarly, the emission spectra (h in Figure 4.12 and 4.13) of the model cations 71 and 72 also reveal a relatively weak fluorescence. Specifically, the quantum yield of 71 and 72 is only ca. 0.1, while that of the models 62 and 67 is ca. 0.7. Thus, the 3H-indolium segment of 53d, 54d, 71 and 72 tends to encourage the nonradiative deactivation of the adjacent BODIPY, presumably, as a result of photoinduced electron transfer. Indeed, the reduction potentials of similar 3H-indolium derivatives suggest that
this cationic fragment can accept an electron from the excited BODIPY, quenching its fluorescence.\textsuperscript{170m,n}

The absorption spectra (\textit{a} and \textit{c} in Figure 4.14) of the dyad \textit{55a} and model BODIPY \textit{68} show bands at 520 and 496 nm respectively. The difference in wavelength indicates that the conjugation of the BODIPY component of \textit{55a} extends over the adjacent 1,2-ethenediyl spacer. In addition to the band at 520 nm, the spectrum of \textit{55a} also shows a weak and broad absorption at 569 nm. This band increases after the addition of acid with the formation of \textit{55d} (\textit{b} in Figure 4.14) and resembles the absorption (\textit{h} in Figure 4.14) of the model cation \textit{73}. These observations suggest that the ring-closed isomer \textit{55a} is in equilibrium with a relatively small fraction of ring-open isomer \textit{55b}. Furthermore, the difference in absorption wavelength between \textit{55a} and either \textit{55b} or \textit{55d} indicates that the 3\textit{H}-indolium and BODIPY fragments of the ring-opened species are in conjugation. This behavior is in contrast with that of \textit{53d} and \textit{54d} and demonstrates that the absence of a 1,4-phenylene spacer between these two subunits permits their coplanar arrangement. A similar effect is also observed with the transformation of \textit{56a} into \textit{56d}. Specifically, the absorption spectrum of \textit{56a} reveals a band for the BODIPY component at 527 nm. This absorption resembles that (\textit{g} in Figure 4.14) of the model \textit{69}. Upon addition of acid, the oxazine ring of \textit{56a} opens to generate \textit{56d} and bring the 3\textit{H}-indolium and catechol ligand in conjugation. The tetrahedral arrangement around the boron center, however, prevents conjugation from extending over to the BODIPY fragment. As a result, the absorption spectra (\textit{f} and \textit{h} in Figure 4.14) of \textit{56d} and the model \textit{74} show a band for the extended 3\textit{H}-indolium cation at 485 nm and one for the BODIPY chromophore at 527 nm.
The emission spectrum of the model 67 shows the typical BODIPY fluorescence at 506 nm with a quantum yield of 0.73. The incorporation of this particular fluorophore within the dyad 55a, however, tends to encourage its nonradiative deactivation. In fact, the fluorescence of 55a is negligible and does not change after the addition of acid with the formation of 55d. Consistently, the model cation 73 is also essentially nonemissive. Similarly, the emission spectra of the dyad 56a recorded before and after the addition of acid and the formation of 56d as well as those of the model compounds 69 and 74 do not reveal any significant fluorescence. Thus, the chelation of the boron center with a catechol ligand quenches effectively the excited state of the BODIPY fluorophore, suppressing its emission.
4.2.3. Time-Resolved Spectroscopy

The laser excitation of the phenoxy fragment, embedded within our photochromic oxazines, at 355 nm generally results in the cleavage of the [C–O] bond at the junction of the two heterocycles with the formation of a phenolate chromophore (Figure 4.1).\textsuperscript{170} Consistently, a ground-state absorption for this particular chromophore appears in the visible region of the absorption spectrum recorded upon illumination. When the substituent in position 4 is a nitro group, this band is centered at 430 nm and decays monoexponentially on a submicrosecond timescale with the spontaneous reformation of the [C–O] bond. In contrast to this behavior, the time-resolved spectrum of the dyad 52a, recorded under otherwise identical irradiation conditions, does not reveal any significant change. Thus, the covalent attachment of a BODIPY fluorophore to position 4 of the phenoxy fragment of the photochromic component prevents the photoinduced opening of the oxazine ring. In fact, all the structural modifications explored so far at this particular position translated in the suppression of the photoinduced ring-opening process with one exception.\textsuperscript{170d,j,n} These observations suggest that the presence of a nitro group on this fragment is essential to facilitate the photochromic transformation.

In contrast to the spectroscopic response of 52a, the time-resolved spectra (a in Figure 4.15 and a, c and d in Figure 4.16) of the dyads 53a–56a show pronounced positive and negative bands in the visible region. In all instances, excitation at 355 nm results in the bleaching of the ground-state absorption of the BODIPY component and the appearance of two bands at ca. 450 and 650 nm. For each dyad, both absorptions decay monoexponentially on a microsecond timescale (e and d in Figure 4.15 and a–f in Figure 4.17), suggesting that they are both associated with the very same species. Curve fitting of the temporal absorbance profiles indicates the lifetime of this transient to be 25, 26, 31 and 10 ms for 53a, 54a, 55a and 56a respectively (τ in Table 4.1) in the absence of molecular oxygen. In aerated solution, instead, the lifetime of this species is drastically reduced. These observations, together with the position and shape of the transient bands,
are consistent with the formation of the triplet state of the BODIPY component.\footnote{198} Indeed, similar transient absorptions can also be observed in the spectra of optically-matched solutions of the model compounds 62 and 67 (\textit{b} in Figure 4.15 and 4.16). Furthermore, the transient bands of 62 and 67 and those of the corresponding dyads 53a and 54a decay with essentially identical kinetics, in agreement with their assignment to a triplet state of the BODIPY component. However, the absorptions detected for the dyads are significantly more intense than those observed for the models, under identical irradiation conditions. Indeed, plots of the absorbance, measured at the end of the excitation pulse, against the laser energy reveal that the population of the triplet state in the dyads 53a and 54a is 7 and 12 times respectively more efficient than in the corresponding models 62 and 67. These observations indicate that the excitation energy of the oxazine component is transferred to the BODIPY component with the concomitant population of its triplet state.\footnote{199} Nonetheless, the excitation spectra (\textit{a} in Figure 4.18 and 4.19) of the emissive dyads 53a and 54a differ from the corresponding absorption spectra (\textit{b} in Figures 4.18 and 4.19) and, instead resemble the absorption spectra (\textit{c} in Figures 4.18 and 4.19) of the BODIPY models 62 and 67 respectively. The lack of any significant contribution of the photochromic component to the emission intensity of the fluorescent fragment excludes the occurrence of energy transfer in the singlet state from the former to the latter. Thus, the ability of the oxazine component to encourage the population of the BODIPY triplet must be a result of triplet–triplet energy transfer. Specifically, the local excitation of the oxazine component is followed by intersystem crossing and then energy transfer to the BODIPY triplet, rather than ring-opening. Consistently, the time-resolved spectra of the dyads 53a–56a do not show the characteristic ground-state absorption associated with the 4-nitrophenolate chromophore\footnote{170} of the ring-open isomers 53b–56b and, instead, reveal the exclusive formation of the BODIPY triplet. In summary, the BODIPY component of these dyads intercepts the excited state of the oxazine component that would otherwise be responsible for ring opening and demonstrates that the photochemistry of this family of photochromic
compounds evolves predominantly through the potential energy surface of their triplet state.

Figure 4.15. Time-resolved absorption spectra (MeCN, 20 °C) of optically-matched ($A_{355} = 0.3$) solutions of 54a (a) and 67 (b) recorded after 0.1 µs from pulsed laser irradiation (355 nm, 6 ns, 12 mJ). Corresponding temporal absorbance evolution for 54a at 450 (c) and 630 nm (d). Absorbance measured at 450 nm upon excitation of 54a (e) and 67 (f) against the laser energy.
Figure 4.16. Time-resolved absorption spectra (MeCN, 20 °C) of optically-matched \(A_{355} = 0.3\) solutions of 53a (a), 62 (b), 55 (c) and 56a (d) recorded after 0.1 µs from pulsed laser irradiation (355 nm, 6 ns, 12 mJ).
Figure 4.17. Temporal absorbance profiles (MeCN, 20 °C) of optically-matched ($A_{355} = 0.3$) solutions of 53a at 430 (a) and 670 nm (b), 55a at 430 (c) and 650 nm (d) and 56a at 450 (e) and 640 nm (f) recorded upon pulsed laser irradiation (355 nm, 6 ns, 12 mJ).
Figure 4.18. Excitation (a, $\lambda_{Em} = 540$ nm) and absorption (b) spectra of 53a and absorption spectrum (c) of 62 (8 $\mu$M, MeCN, 20 °C).

Figure 4.19. Excitation (a, $\lambda_{Em} = 585$ nm) and absorption (b) spectra of 54a and absorption spectrum (c) of 67 (23 $\mu$M, MeCN, 20 °C).

4.3. Conclusions

A photochromic oxazine can be connected to either the $\pi$-system or the boron center of a BODIPY fluorophore through five different unsaturated spacers to produce fluorophore–photochrome dyads in one to four synthetic steps. In the resulting assemblies, the two functional components are electronically isolated in the ground state and their absorption spectra reveal essentially the same band observed for model BODIPY fluorophores. Similarly, comparison of the emission spectra of the dyads and their models show that the
oxazine photochrome has a negligible influence on the fluorescence of the BODIPY component with one exception. On the contrary, the photochromism of the oxazine component is essentially suppressed, after its covalent connection to the BODIPY fluorophore, in all dyads. In fact, intersystem crossing dominates the excitation dynamics of these molecular constructs and prevents the photoinduced opening of the oxazine ring. Specifically, the local excitation of the oxazine component is followed by intersystem crossing and then energy transfer to the BODIPY component instead of ring opening. Thus, these observations demonstrate that the photoinduced opening of this family of photochromic oxazines occurs along the potential energy of their triplet state. Indeed, my results provide, for the very first time, conclusive evidence on the role of the triplet state in the photochromism of these compounds and, therefore, can guide the structural design of novel members of this class of photoresponsive molecules.
CHAPTER 5

FAST FLUORESCENCE SWITCHING WITHIN HYDROPHILIC SUPRAMOLECULAR ASSEMBLIES

5.1. Background

The ability to switch fluorescence under optical control can offer the opportunity to monitor the translocation of labeled targets within a biological sample and provide valuable information on the fundamental factors governing the diffusion of biorelevant species.\textsuperscript{13–24} Similarly, fluorescence photoswitching can permit the separation in time of spatially-indistinguishable probes and allow the reconstruction of images with subdiffraction resolution.\textsuperscript{25–42} As a result, photoswitchable fluorophores are becoming invaluable analytical tools in the biomedical laboratory for the investigation of cellular dynamics and the visualization of cellular substructures. Indeed, the significant implications that such molecular switches can have in biomedical research, together with the need to advance our basic understanding on the excitation dynamics of organic chromophores, are encouraging the identification of practical mechanisms to activate fluorescence under the influence of optical stimulations.\textsuperscript{7–12} In this context, photochromic compounds\textsuperscript{82–87} can be valuable components for the assembly of photoswitchable fluorescent constructs. In fact, their photoinduced and reversible transformations can be engineered to regulate the emission of fluorescent partners.\textsuperscript{88–94} Specifically, the integration of fluorescent and photochromic components within the same molecular or supramolecular assembly can be exploited to modulate the emission of the former by operating the latter with optical inputs. Generally, electron or energy transfer processes are invoked to establish communication between the functional components in the excited state and manipulate the excitation dynamics of the emissive species.\textsuperscript{105–127} In particular, the excited fluorophore can be designed to exchange an electron with or transfer energy to only one of the two interconvertible states of the photochromic component. Under these conditions, the photochromic transformation
activates or suppresses fluorescence reversibly. Indeed, numerous examples of fluorophore–photochrome constructs have already been developed successfully on the basis of these mechanisms.\textsuperscript{88–94}

Photochromic spiropyrans undergo ring-opening reactions, followed by \textit{cis} $\rightarrow$ \textit{trans} isomerizations, upon ultraviolet irradiation to produce merocyanine chromophores.\textsuperscript{200–211} The photogenerated isomers revert back to the original species either thermally, over the course of hundreds of seconds, or photochemically, upon visible illumination. Such reversible structural transformations are accompanied by pronounced absorbance changes in the visible region as well as by significant shifts in redox potentials. In the presence of compatible fluorescent partners, the photoinduced modification of these spectroscopic and electrochemical parameters can activate energy and electron transfer pathways respectively and quench fluorescence.\textsuperscript{88–94} On the basis of these operating principles, we have been able to modulate the emission of a diversity of fluorophores in liquid solutions\textsuperscript{182} and within rigid matrices.\textsuperscript{183} In search of strategies to impose biocompatibility on these photoswitchable systems, I then devised a supramolecular protocol to transport mixtures of borondipyrromethene (BODIPY) fluorophores and spiropyran photochromes within the intracellular environment and operate them successfully within live cells.\textsuperscript{184} My method is based on the encapsulation of separate fluorescent and photochromic components within the hydrophobic interior of appropriate polymer micelles. Nonetheless, the slow switching speeds and modest fatigue resistances of the photochromic components limit significantly the performance of these photoswitchable supramolecular assemblies. Specifically, a single switching cycle requires more than $10^4$ s and significant photodegradation occurs within few cycles.

In parallel to our investigations on fluorescence photoswitching with spiropyrans,\textsuperscript{182–184} our group developed a new family of photochromic compounds with fast switching speeds and excellent fatigue resistances.\textsuperscript{170} They are based on the photoinduced opening
and thermal closing of an oxazine ring. These photochromic switches can complete a single cycle on a submicrosecond timescale and tolerate hundreds of cycles with no sign of degradation even in air. In fact, our group has been able to modulate the emission of BODIPY and coumarin fluorophores with microsecond switching speeds and for hundreds of cycles on the basis of such photochromic transformations.\textsuperscript{170m–o,q} As a result, our group envisaged the possibility of introducing appropriate members of this family of photochromic compounds within our biocompatible supramolecular constructs, in place of their spiropyran components, with the ultimate goal of improving their performance. In this article, I report the design and synthesis of two hydrophobic photochromic oxazines, their introduction within biocompatible polymer micelles as well as the photochemical and photophysical properties of the resulting supramolecular assemblies and appropriate model compounds.

5.2. Results and Discussion

5.2.1. Design and Synthesis

The co-polymer 51 (Figure 5.1) has multiple hydrophobic and hydrophilic side chains along a common poly(methacrylate) backbone. In aqueous environments, this particular amphiphilic macromolecule forms micellar assemblies with a hydrodynamic diameter of ca. 40 nm that are capable of trapping organic dyes in their hydrophobic interior.\textsuperscript{184} Specifically, the BODIPY fluorophore 77 can be embedded within these supramolecular constructs together with a spiropyran photochrome. The photoinduced transformation of the spiropyran into the corresponding merocyanine activates electron and energy transfer pathways from the excited fluorophore to the photochrome that encourage the nonradiative deactivation of the former. In principle, the spiropyran component can be replaced with the photochromic oxazine 78a (Figure 5.2) to enhance the switching speeds and fatigue resistance of the overall fluorescent construct. Indeed, the oxazine ring of this particular compound opens to produce the zwitterionic isomer 78b in less than 6 ns
upon ultraviolet illumination in acetonitrile. The photogenerated isomer reverts spontaneously to the original one in less than 10 ms, under these experimental conditions, and the photochromic system can be switched back and forth between its two states for hundreds of cycles with no sign of degradation. The photoinduced transformation of 78a into 78b brings the 4-dimethylaminostyryl appendage in conjugation with the resulting 3H-indolium cation. This extended chromophoric fragment absorbs in the same range of wavelengths where 77 emits and, therefore, can accept the excitation energy of this fluorophore. Furthermore, the redox potentials of similar compounds indicate that the transfer of an electron from the excited fluorophore to the 3H-indolium chromophore is exergonic with a free energy change of –0.5 eV. Thus, both electron and energy transfer pathways can be activated with the photoinduced transformation of 78a into 78b to quench the emission of 77, if fluorescent and photochromic components are both trapped within the same supramolecular assembly. On the basis of these considerations, I envisaged the possibility of appending an oligo(methylene) tail to this photochromic oxazine, in the form of compound 79a (Figure 5.2), to encourage the entrapment of the resulting molecule within micelles of the amphiphilic co-polymer 51. In particular, I prepared 79a in two synthetic steps (Figure 5.3), starting from commercial and known precursors.

![Figure 5.1. Amphiphilic co-polymer 51 and hydrophobic fluorophore 77.](image)
Figure 5.2. Photoinduced and reversible transformation of the oxazines 39a, 78a–81a into the corresponding zwitterionic isomers 39b, 78b–81b.

Figure 5.3. Synthesis of 79a.
The integration of 77 and 79a within the same micellar construct can offer the opportunity to suppress the emission of the former with the photoinduced ring opening of the latter. A similar photochemical transformation, however, can also be exploited to activate, rather than deactivate, fluorescence. Specifically, the photoinduced opening of the oxazine ring of 39a (Figure 5.2) brings its coumarin appendage in conjugation with the 3H-indolium cation of 39b and shifts the main absorption band of this fluorescent appendage from 410 to 570 nm in acetonitrile. As a consequence, illumination at 532 nm results in significant fluorescence only after the photoinduced opening of the oxazine ring. Thus, the introduction of such fluorophore–photochrome dyad within micellar assemblies of the amphiphilic co-polymer 51 can translate into the realization of photoactivatable and biocompatible fluorescent constructs. Therefore, I designed an analog of 39a with a pendant oligo(methylene) tail, in the form of compound 80a (Figure 5.2), to facilitate the encapsulation of the resulting dyad within micelles of 51. In particular, I prepared 80a in four synthetic steps (Figure 5.4), starting from commercial and known precursors. Following a similar synthetic procedure (Figure 5.5), I also prepared the hydrophilic analog 81a (Figure 5.2). Its pendant oligo(ethylene glycol) tail is designed to impose aqueous solubility on the fluorophore–photochrome dyad and, therefore, permit the investigation of its photochemical and photophysical properties in aqueous solutions lacking any micellar host.
Figure 5.4. Synthesis of 80a.
Figure 5.5. Synthesis of 81a and the hexafluorophosphate salt of 83.

5.2.2. Steady-State Spectroscopy

The absorption spectra \((a\text{ and } b\text{ in Figure 5.6})\) of acetonitrile solutions of 78a and 79a are very similar, indicating that the hydrophobic tail of the latter has a negligible influence on the photophysical properties of the heterocyclic core. In both instances, the spectrum shows bands at 305 and 555 nm that correspond to the 4-nitrophenoxy fragment of the ring-closed isomer and the 3H-indolium cation of the ring-open species respectively. Indeed, the absorption in the visible region resembles that of the model compound 82 \((c\text{ in Figure 5.6})\), which has essentially the same chromophoric fragment of the ring-open isomers 78b and 79b. Thus, the two isomers of each system co-exist under these experimental conditions with a ratio of 90:10 in favor of the ring-closed species.
The two oxazines 78a and 79a are relatively hydrophobic and, as a result, they are sparingly soluble in aqueous solutions. However, they readily dissolve in neutral phosphate buffer saline (PBS) in the presence of the amphiphilic co-polymer 51. The absorption spectra of the resulting dispersions (d and e in Figure 5.6) show predominantly bands at 390 and 560 nm for the 4-nitrophenolate anion and 3H-indolium cation respectively of the zwitterionic species 78b and 79b. The pH dependence of the
absorption spectrum (a and b in Figure 5.7) of 4-nitrophenol confirms the assignment of the band at 390 nm to the anionic chromophore of the ring-open isomers 78b and 79b. Indeed, this band resembles the absorption observed for the 4-nitrophenolate anion at a pH of 8.0 (b in Figure 5.7 S4). These observations suggest that both molecular switches are partially exposed to the aqueous medium, despite their interactions with the amphiphilic co-polymer 51. Indeed, the ring-open isomers are significantly more polar than their ring-closed counterparts and, as a result, the transition from organic to aqueous environments tends to encourage their population. Specifically, the absorbance of the 3H-indolium chromophore in the visible region indicates that the ratio between 78a and 78b is 30:70, while that between 79a and 79b is 85:15.212,213 The different ratios suggest that the two molecular switches reside in different domains within their polymeric host. Presumably, the hydrophobic tail of one system limits the exposure of the heterocyclic core to the polar aqueous environment and, therefore, decreases the fractional concentration of ring-open isomer relative to that associated with the other system.

![Absorption Spectrum](image)

**Figure 5.7.** Absorption spectra of solutions (0.1 mM, PBS, 25 °C) of 4-nitrophenol with a pH of 6.0 (a) and 8.0 (b).

In agreement with my design logic, the absorption band of the 3H-indolium chromophore of the ring-open isomers 78b and 79b is, indeed, positioned in the same range of wavelengths where 77 emits (f in Figure 5.6).184 This pronounced spectral overlap
together with the redox potentials of model compounds\textsuperscript{170m} suggest that the concomitance of energy and electron transfer pathways can quench the fluorescence of \textit{77}, if this component and either one of the two molecular switches are trapped within the same micellar assembly. Nonetheless, the fraction of ring-open isomer is relatively large for both systems, under these experimental conditions. This unexpected complication prevents the implementation of our operating principles for photoinduced fluorescence suppression, which instead require a significant population of the quenching species only after ultraviolet illumination.

The absorption spectra (\textit{a} and \textit{b} in Figure 5.8) of acetonitrile solutions of \textit{39a} and \textit{80a} show a band at 410 nm for the coumarin appendage of the ring-closed isomer,\textsuperscript{170a} but do not reveal any significant absorbance for the \textit{3H}-indolium cation of the ring-open species. Indeed, the spectrum (\textit{c} in Figure 5.8) of the model compound \textit{49} indicates that this particular cationic chromophore absorb at 570 nm. In analogy to \textit{78a} and \textit{79a}, also \textit{39a} and \textit{80a} are relatively hydrophobic and sparingly soluble in water, but can readily be dispersed in aqueous solution together with the amphiphilic co-polymer \textit{51}. In contrast to \textit{78a} and \textit{39a}, however, the spectra (\textit{d} and \textit{e} in Figure 5.8) of the resulting aqueous dispersions are very similar to those of the acetonitrile solutions. Once again, only the band for the coumarin appendage of the ring-closed isomers \textit{39a} and \textit{80a} can be observed at 400 nm. Similarly, the absorption spectrum (\textit{a} in Figure 5.9) of an acetonitrile solution of their hydrophilic analog shows exclusively a band at 405 nm for the ring-closed isomer \textit{81a}. However, an additional band is clearly present at 610 nm in the spectrum (\textit{b} in Figure 5.9) of the very same species dissolved in PBS in the absence of \textit{51}. This band resembles that (\textit{c} in Figure 5.9) of the model \textit{3H}-indolium \textit{83} (Figure 5.10) and indicates the co-existence of \textit{81a} and \textit{81b} in a ratio of 90:10 under these conditions.\textsuperscript{213,214} These observations demonstrate that the micellar host protects effectively \textit{39a} and \textit{80a} from the aqueous environment and discourages the population of their ring-open isomers \textit{39b} and \textit{80b}. Thus, the supramolecular encapsulation of these fluorophore–photochrome dyads
within the hydrophobic interior of the amphiphilic co-polymer offers the opportunity to implement our operating principles for fluorescence photoactivation, which demand the population of the emissive isomer only after ultraviolet stimulations. Indeed, the photoinduced opening of the oxazine ring of 39a and 80a within the micellar assemblies can extend the conjugation of the coumarin fluorophore within the photogenerated isomers 39b and 80b to produce a 3H-indolium chromophore able to emit at 645 nm, according to the emission spectrum of the model compound 49 (f in Figure 5.8).\textsuperscript{215}

![Figure 5.8](image-url)

**Figure 5.8.** Absorption spectra of solutions (MeCN, 25 °C) of 39a (a, 5 µM), 80a (b, 5 µM) and the hexafluorophosphate salt of 49 (c, 1 µM). Absorption spectra of dispersions (PBS, pH = 7.0, 25 °C) of 1 (50 µg mL\(^{-1}\)) and either 39a (d, 5 µg mL\(^{-1}\)) or 80a (e, 5 µg mL\(^{-1}\)). Emission spectrum (f) of a solution (MeCN, 25 °C, \(\lambda_{\text{Ex}} = 530\) nm) of the hexafluorophosphate salt of 49 (10 µM).
5.2.3. Time-Resolved Spectroscopy

The illumination of an aqueous dispersion of 51 and either 39a or 80a at 355 nm with a pulsed laser opens the oxazine ring of the molecular switch, entrapped within the polymeric construct, to generate the zwitterionic isomer 39b or 80b within the laser pulse (6 ns). Consistently, the absorption spectrum (a in Figures 5.11 and 5.12) of the dispersion, recorded 0.1 ms after illumination, shows the appearance of band at 620 nm. This transient absorption resembles the steady-state band (c in Figure 5.8) observed for
the model compound 82 and corresponds to a ground-state absorption of the 3H-indolium chromophore of the ring-open isomer 39b or 80b. Essentially the same transient absorption is observed also in the spectrum (a in Figure 5.13) of an aqueous solution of the hydrophilic analog 81a in the absence of the polymeric host, under otherwise identical conditions. However, the dependence of the absorbance of this band on the laser intensity indicates the quantum yield for the photochromic transformation to be ca. 0.02 for 39a and 80a within the micellar assemblies and only 0.006 for 81a in water. These values suggest that the polymeric envelope around the photochromic components limits their exposure to the aqueous environment, in agreement with the indications emerged from the analysis of the steady-state spectra. Furthermore, the quantum yield for the photochromic transformation of 39a and 80a in the micellar assemblies is identical to that measured for 39a in acetonitrile170 suggesting that the polymeric host provides an environment similar to this particular organic solvent.
Figure 5.11. Absorption spectrum (a) of a dispersion (H$_2$O, 25 °C) of 51 (2 mg mL$^{-1}$) and 80a (9 µg mL$^{-1}$), recorded 0.1 µs after pulsed illumination (355 nm, 6 ns, 15 mJ). Emission spectra of the same dispersion, recorded upon pulsed irradiation at 532 nm (6 ns, 30 mJ) without (b) and with (c) simultaneous pulsed illumination at 355 nm. Absorbance evolution at 620 nm of the same dispersion upon pulsed irradiation at 355 nm with the corresponding monoexponential fitting (d). Relative emission intensity at 650 nm of the same dispersion, recorded upon pulsed irradiation at 532 nm without and with simultaneous pulsed illumination at 355 nm (e).
Figure 5.12. Absorption spectrum (a) of a dispersion (H₂O, 25 °C) of 51 (2 mg mL⁻¹) and 39a (9 µg mL⁻¹), recorded 0.1 µs after pulsed illumination (355 nm, 6 ns, 15 mJ). Emission spectra of the same dispersion, recorded upon pulsed irradiation at 532 nm (6 ns, 30 mJ) without (b) and with (c) simultaneous pulsed illumination at 355 nm. Absorbance evolution at 620 nm of the same dispersion upon pulsed irradiation at 355 nm with the corresponding monoexponential fitting (d).
Figure 5.13. Absorption spectrum (a) of a solution (30 µM, H₂O, 25 °C) of 81a, recorded 0.1 µs after pulsed illumination (355 nm, 6 ns, 15 mJ). Emission spectra of the same solution, recorded upon pulsed irradiation at 532 nm (6 ns, 30 mJ) without (b) and with (c) simultaneous pulsed illumination at 355 nm. Absorbance evolution at 620 nm of the same dispersion upon pulsed irradiation at 355 nm with the corresponding monoexponential fitting (d).

The isomers 39b and 80b, photogenerated within the polymer micelles, as well as the ring-open species 81b, produced in the absence of the polymeric host, all revert spontaneously back to the ring-closed species with first-order kinetics. As a result, the absorbance for their 3H-indolium chromophore decays monoexponentially (d in Figures 5.11-5.13). Curve fitting of the temporal absorbance profiles indicates the lifetime of these species to be ca. 0.2 ms in all instances. This value is essentially identical to that of 39b measured in acetonitrile. Furthermore, all three photochromic systems can be
switched back and forth between their two states for hundreds of cycles with no sign of
degradation. Thus, the environment around the photochromic components has negligible
influence on their reisomerization kinetics and fatigue resistances.

The illumination of 39a and 80a within the polymer micelles and of 81a in the absence of
the polymeric host at 532 nm does not result in any significant fluorescence (b in Figures
6, S6 and S7). Indeed, the coumarin fluorophore of 39a, 80a and 81a does not absorb at
this particular wavelength and, therefore, it cannot emit. After the photoinduced ring-
opening process, however, the coumarin absorption shifts from 400 to 620 nm (a in
Figures 5.11-5.13). Therefore, the fluorescent appendage within the ring-open isomers
39b, 80b and 81b can absorb the exciting radiations at 532 nm and emit as a result. In
fact, the simultaneous illumination of the sample at 355 nm, to open the oxazine ring of
39a, 80a and 81a, and at 532 nm, to excite the coumarin fluorophore of 39b, 80b and
81b, is accompanied by the appearance of an intense band at 650 nm in the corresponding
emission spectrum (c in Figures 5.11-5.13). This band disappears after the spontaneous
reisomerization of 39b, 80b and 81b back to 39a, 80a and 81a. As a consequence, the
emission intensity at 650 nm can be switched on and off for multiple cycles (e in Figure
5.11) simply by turning on and off an excitation source at 355 nm, while illuminating the
sample at 532 nm.

5.3. Conclusions

Hydrophobic fluorophore–photochrome dyads can be dissolved in aqueous environments
with the assistance of an amphiphilic polymer. In the resulting supramolecular
assemblies, the photochromic component retains its photochemical properties essentially
unaltered. Specifically, its oxazine ring opens upon ultraviolet illumination to bring the
adjacent coumarin fluorophore in conjugation with a 3H-indolium cation. This structural
transformation shifts the main absorption of the fluorophore to the visible region. As a
result, the visible illumination of aqueous dispersions of such nanostructured constructs
results in significant fluorescence only under concomitant ultraviolet irradiation. Furthermore, the photochromic component reverts to the original state on a microsecond timescale after the spontaneous closing of its oxazine ring. In fact, the fluorescence of these supramolecular assemblies can be modulated for hundreds of cycles with microsecond switching speeds simply by turning on and off an ultraviolet source under visible illumination. Thus, our operating principles for fluorescence modulation under optical control can translate into the realization of switchable probes compatible with aqueous environments and, ultimately, lead to valuable analytical tools for the investigation of biological samples.
CHAPTER 6

EXPERIMENTAL PROCEDURES

6.1. Materials and Methods

Chemicals were purchased from commercial sources and used as received with the exception of MeCN and CH$_2$Cl$_2$, which were distilled over CaH$_2$. Compounds 16, 17, 18, 23–26, the iodide salt of 24, 35, 36a, 39a, 44, 45, 46, 49, 51, 57, 62, 66, 68, 70, 77, 78a, 82 and 85 were prepared according to literature procedures. All reactions were monitored by thin-layer chromatography, using aluminum sheets coated with silica (60, F$_{254}$). Electrospray ionization mass spectra (ESIMS) were recorded with a Bruker micrOTO-Q II spectrometer. Fast atom bombardment mass spectra (FABMS) were recorded with a VG Mass Lab Trio-2 spectrometer in a 3-nitrobenzyl alcohol matrix. Nuclear magnetic resonance (NMR) spectra were recorded with Bruker Avance 400 and 500 spectrometers.

Differential pulse voltammograms were recorded with a CH Instruments 660 electrochemical analyzer under Ar, using a glassy-carbon working electrode (3 mm), a platinum counter electrode and a Ag/AgCl reference electrode.

6.2. Absorption and Emission Spectroscopies

Steady-state absorption spectra were recorded with a Varian Cary 100 Bio spectrometer, using quartz cells with a path length of 0.5 or 1.0 cm. Steady-state emission spectra were recorded with a Varian Cary Eclipse spectrometer in aerated solutions. Fluorescence quantum yields were determined with fluorescein and rhodamine B standards, following a literature protocol.$^1$ Time-resolved absorption and emission spectra were recorded with a Luzchem Research mLFP–111 spectrometer in aerated solutions by illuminating orthogonally the sample with a Continuum Surelite II–10 Nd:YAG pulsed laser. For
absorption measurements, the laser was operated at 355 nm (10 mJ) and the transmittance was measured in the 350–700 nm spectral range. For fluorescence measurements, the laser was operated at 355 (10 mJ) for 36a, 37a, 39a, and for dispersions of 51 doped with 39a, 80a and 81a or simultaneously at 355 (10 mJ) and 532 nm (30 mJ) for 39a and the emission intensity was measured in the 400–800 nm spectral range. The quantum yields for the photochromic transformations were determined with a benzophenone standard, following a literature protocol. PMMA ($M_W = 12 \times 10^4$) films doped with 39a were prepared by spin coating CH$_2$Cl$_2$ solution of dopant (1.0 mg mL$^{-1}$) and polymer (52 mg mL$^{-1}$) on a glass plate at 420 rpm for 9 s. The thicknesses (6 µm) of the resulting films were measured with a Tencor Instruments 10-00090 surface profilometer.

6.3. Single-Molecule Imaging and Spectroscopy

PVA ($M_W = 1 \times 10^4$) films doped with 39a or 49 were prepared by spin coating an aqueous solution of dopant (0.2–0.7 nM) and polymer (1 mg mL$^{-1}$) on a glass plate at 3000 rpm for 45 s. PMMA ($M_W = 35 \times 10^4$) films doped with 39a or 49 were prepared by spin coating a PhMe solution of dopant (0.2–1.0 nM) and polymer (4.3 mg mL$^{-1}$) on a glass plate at 3000 rpm for 45 s. Polymer nanoparticles doped with 39a were prepared by thoroughly mixing a CHCl$_3$ solution of the dye (0.1 mg mL$^{-1}$, 50 µL) with a solution of the polymer (2.5 mg mL$^{-1}$, 100 µL) in the same solvent. Then, the mixture was gently flushed with N$_2$ to evaporate the solvent, and the residue was dispersed in PBS buffer (1 mL, pH = 7.0) with vigorous shaking. The resulting dispersion was filtered and a portion (20 µL) of the filtrate was deposited onto a cover glass and imaged with the apparatus described in the following section, after the evaporation of the solvent in air. Another aliquot of the filtrate was diluted ca. 100 times, deposited onto a TEM grid and imaged with a Philips 120 kV BioTwin microscope, after the evaporation of the solvent in air.

Fluorescence images were recorded with a custom-built wide-field epi-fluorescence microscope. Excitation was preformed with a Coherent VERDI V5 continuous-wave
(CW) laser at 532 nm and the isomerization was photoinduced with a Cobolt AB Zouk CW laser at 355 nm. The lasers were combined in one beam with a dichroic mirror and illumination was performed in the wide-field mode by focusing both lasers in the back focal plane of a Leica HCX PL APO 100×/1.4 oil immersion objective lens. A large excitation (and activation) spot (ca. 30 × 30 μm²) was obtained. The fluorescence signal was collected by the same objective (similar to an inverted microscope), separated from activation and excitation light by another dichroic mirror and then detected by an Andor Technology IXON-DU-897 EMCCD camera. In order to minimize background and optimize localization, the frame rate of the camera was adjusted to 10 ms. Typically, sets of 50,000–90,000 frames were collected and localization of single-molecule events and image reconstruction were performed as previously described. The intensity of the activation laser was set to a low value (typically < 1 W cm⁻²) initially and then it was slightly increased during the measurement (up to ca. 100 W cm⁻²).

6.4. Experimental Procedures

Synthesis of 8a

A solution of 16 (100 mg, 0.3 mmol), 17 (151 mg, 1 mmol) and TFA (0.5 mL, 7 mmol) in MeCN (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH₂Cl₂ (5 mL). The addition of Et₂O (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH₂Cl₂ (20 mL) and washed with H₂O (20 mL). The organic phase was dried over Na₂SO₄ and the solvent was distilled off under reduced pressure to give 8a (50 mg, 35%) as a purple solid. ESIMS: m/z = 444.2287 [M + H]⁺ (m/z calcd. for C₂₇H₃₀N₃O₃ = 444.2282); ¹H NMR (CDCl₃): δ = 1.10 (3H, t, 8 Hz), 1.73 (6H, s), 2.25 (3H, s), 2.42–2.48 (5H, m), 5.24 (2H, s), 6.44 (1H, d, 9 Hz), 7.25–7.29 (2H, m), 7.38–7.43 (2H, m), 7.64 (1H, d, 15 Hz), 7.77 (1H, s), 7.84–7.91 (2H, m).
Synthesis of 9a

A solution of 16 (155 mg, 0.5 mmol), 18 (191 mg, 0.7 mmol) and TFA (0.5 mL, 7 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure to give 9a (119 mg, 42%) as a purple solid. ESIMS: $m/z = 566.2445$ [M + H]$^+$ ($m/z$ calcd. for C$_{37}$H$_{32}$N$_3$O$_3$ = 566.2438); $^1$H NMR (CDCl$_3$): $\delta = 1.41$ (6H, bs), 4.60 (2H, s), 6.24 (1H, d, 16 Hz), 6.63 (1H, d, 8 Hz), 6.75 (1H, d, 16 Hz), 6.86–6.89 (2H, m), 7.00–7.15 (10H, m), 7.24–7.29 (6H, m), 7.96–8.02 (2H, m); $^{13}$C NMR (CDCl$_3$): $\delta = 24.8, 41.2, 50.5, 109.2, 118.1, 119.7, 120.4, 122.7, 123.5, 123.6, 123.8, 124.5, 125.1, 125.5, 126.4, 126.7, 128.1, 128.2, 129.8, 130.0, 130.1, 131.7, 138.7, 140.9, 146.8, 147.6, 190.9.

Synthesis of 10a

A solution of 16 (300 mg, 1.0 mmol), 19 (89 $\mu$L, 1.0 mmol) and TFA (100 $\mu$L, 1.4 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure to give 10a (100 mg, 26%) as a yellow solid. ESIMS: $m/z = 405.1267$ [M + H]$^+$ ($m/z$ calcd. for C$_{23}$H$_{21}$N$_2$O$_3$S = 405.1267); $^1$H NMR (CDCl$_3$): $\delta = 1.28$ (3H, bs), 1.59 (3H, bs), 4.59 (2H, s), 6.64 (1H, dd, 3 and 8 Hz), 6.87–6.92 (3H, m), 6.96–7.02 (2H, m), 7.10–7.15 (2H, m), 7.23 (1H, d, 5 Hz), 7.28 (1H, s), 7.97–8.01 (1H, m), 8.03 (1H, d, 3 Hz); $^{13}$C NMR (CDCl$_3$): $\delta = 19.1, 23.1, 26.9, 41.1, 50.6, 103.9, 109.2, 118.1, 120.4, 121.2, 122.7, 123.7, 124.5, 126.0, 127.9, 128.1, 129.6, 138.5, 141.1, 146.7, 159.5.
Synthesis of 11a

A solution of 16 (250 mg, 0.8 mmol), 20 (156 mg, 0.8 mmol) and TFA (60 µL, 0.8 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH₂Cl₂ (5 mL). The addition of Et₂O (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH₂Cl₂ (20 mL) and washed with H₂O (20 mL). The organic phase was dried over Na₂SO₄ and the solvent was distilled off under reduced pressure to give 11a (100 mg, 26%) as a purple solid. ESIMS: m/z = 487.1144 [M + H]+ (m/z calcd. for C₂₇H₂₃N₂O₃S₂ = 487.1145); ¹H NMR (CDCl₃): δ = 1.57 (6H, bs), 4.63 (2H, s), 6.19 (1H, d, 5 Hz), 6.63 (1H, d, 4 Hz), 6.88 (4H, t, 7 Hz), 7.02–7.05 (2H, m), 7.10–7.18 (3H, m), 7.22 (1H, d, 4 Hz), 7.99 (1H, dd, 2 and 9 Hz), 8.03 (1H, s); ¹³C NMR (CDCl₃): δ = 18.9, 25.8, 52.3, 54.1, 110.9, 115.1, 116.9, 120.9, 123.3, 125.1, 126.4, 127.7, 129.8, 135.9, 139.2, 140.7, 141.9, 143.4, 146.8, 148.5, 164.0.

Synthesis of 12a

A solution of 16 (180 mg, 0.6 mmol), 21 (65 mg, 0.6 mmol) and TFA (0.29 mL, 6.5 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH₂Cl₂ (5 mL). The addition of hexane (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH₂Cl₂ (20 mL) and washed with H₂O (20 mL). The organic phase was dried over Na₂SO₄ and the solvent was distilled off under reduced pressure. The residue was purified by preparative thin-layer chromatography [SiO₂: AcOEt/hexanes (3:7, v/v)] to give 12a (100 mg, 42%) as a yellow oil. ESIMS: m/z = 405.1266 [M + H]+ (m/z calcd. for C₂₃H₂₀N₂O₃S = 405.1267); ¹H NMR (CDCl₃): δ = 1.57 (6H, s), 4.59 (2H, s), 5.31 (1H, s), 6.18 (1H, d, 16 Hz), 6.62 (1H, d, 7 Hz), 6.79–6.89 (3H, m), 7.12–7.14 (1H, t, 4 Hz), 7.23 (1H, bs), 7.25–7.27 (1H, bs), 7.30 (1H, d, 3 Hz), 7.98–8.02 (2H, m).
Synthesis of 13a

A solution of 16 (150 mg, 0.5 mmol), 22 (104 mg, 0.5 mmol) and TFA (0.24 mL, 6.5 mmol) in EtOH (10 mL) was heated under reflux and Ar for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of hexanes (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure. The residue was dissolved in EtOAc (10 mL) and filtered through a SiO$_2$ plug. The plug was washed with EtOAc (100 mL) and the solvent of the filtrate was distilled off under reduced pressure to give 13a (100 mg, 40%) as a dark-green solid. ESIMS: $m/z = 507.1387 \ [M + H]^+ \ (m/z \ \text{calcd. for } C_{29}H_{26}FeN_2O_3 = 507.1366)$; $^1$H NMR (CD$_3$CN): $\delta = 1.38$ (6H, bs), 3.95 (5H, s), 4.42 (2H, s), 4.46 (2H, s), 4.71 (2H, s), 5.46 (1H, s), 6.05 (1H, d, 16 Hz), 6.70–7.20 (5H, m), 8.01 (1H, bs), 8.13 (1H, bs).

Synthesis of the Hexafluorophosphate Salt of 14a

A mixture of 23 (142 mg, 0.4 mmol) and the iodide salt of 24 (100 mg, 0.3 mmol) in EtOH (20 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (50 mL) caused the precipitation of a solid, which was filtered off, and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 14a (100 mg, 46%) as an orange powder. ESIMS: $m/z = 511.2760 \ [M – PF_6]^+ \ (m/z \ \text{calcd. for } C_{36}H_{35}N_2O = 511.2744)$; $^1$H NMR (CDCl$_3$): $\delta = 0.78$ (3H, s), 1.56 (3H, s), 1.71 (6H, s), 4.22 (3H, s), 4.53 (1H, d, 18 Hz), 5.02 (1H, d, 18 Hz), 6.72–6.82 (2H, m), 6.90 (1H, d, 8 Hz), 7.07 (2H, q, 8 Hz), 7.25–7.40
(3H, m), 7.44 (4H, s), 7.55–7.64 (5H, m), 8.06 (1H, d, 16 Hz), 8.50 (1H, s); $^{13}$C NMR (CDCl$_3$): $\delta$ = 18.2, 25.7, 27.4, 34.2, 40.6, 49.8, 52.7, 105.5, 109.7, 110.6, 115.1, 119.0, 121.0, 122.7, 123.1, 127.6, 128.0, 128.5, 128.9, 129.3, 129.6, 129.7, 130.9, 131.0, 136.6, 138.0, 142.6, 143.9, 147.7, 154.4, 159.2, 182.7, 205.2, 205.7, 206.1.

**Synthesis of 15a**

An aqueous solution of formaldehyde (37%, 0.81 mL, 10 mmol) was added dropwise to a suspension of 25 (880 mg, 5 mmol) in HCl$_{\text{conc}}$ (8 mL). The mixture was stirred at 40 °C for 4 hours and, after cooling down to ambient temperature, diluted with H$_2$O (20 mL). The resulting precipitate was filtered off, washed with H$_2$O (20 mL) dissolved in MeCN (20 mL) together with 26 (721 mg, 3 mmol). The mixture was heated for 24 hours under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (20 mL). The resulting solution was washed with H$_2$O (2 × 20 mL). The solvent of the organic phase was distilled off under reduced pressure and the residue was purified by column chromatography [SiO$_2$: CH$_2$Cl$_2$] to afford 15a (650 mg, 16%) as a white solid. ESIMS: $m/z = 410.1751$ [M + H]$^+$ ($m/z$ calcd. for C$_{27}$H$_{24}$NO$_3$ = 410.1747); $^1$H NMR (CDCl$_3$): $\delta$ = 0.85 (3H, s), 1.61 (3H, s), 2.28 (3H, s), 4.51 (1H, d, 16 Hz), 4.99 (1H, d, 16 Hz), 6.05 (1H, s), 6.79 (2H, d, 8 Hz), 6.89 (1H, t, 8 Hz), 7.14–7.18 (2H, m), 7.26 (1H, d, 8 Hz), 7.32–7.41 (3H, m), 7.67 (2H, bs); $^{13}$C NMR (CDCl$_3$): $\delta$ = 18.8, 18.9, 19.1, 37.2, 104.8, 108.5, 109.1, 109.8, 111.8, 111.9, 113.4, 114.3, 120.5, 128.5, 128.9, 129.1, 129.2, 136.5, 137.9, 147.7, 151.9, 153.4, 157.0, 161.4.

**Synthesis of the Hexafluorophosphate Salt of 27**

A solution of 17 (200 mg, 0.9 mmol) and the iodide salt of 24 (240 mg, 0.8 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was
dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 27 (235 mg, 65%) as a purple solid. FABMS: $m/z = 307$ [M – PF$_6$]$^+$ ($m/z$ calcd. for C$_{21}$H$_{27}$N$_2$ = 307); $^1$H NMR (CDCl$_3$): $\delta = 1.08$ (3H, t, 7 Hz), 1.71 (6H, s), 2.45 (3H, s), 2.42 (2H, q, 7 Hz), 2.64 (3H, s), 4.00 (3H, s), 7.17 (1H, d, 8 Hz), 7.30 (1H, d, 9 Hz), 7.37–7.45 (2H, m), 7.50 (1H, d, 15 Hz), 7.66 (1H, d, 15 Hz), 12.47 (1H, bs); $^{13}$C NMR (CDCl$_3$): $\delta = 10.2, 12.3, 15.0, 17.7, 28.6, 32.3, 50.0, 98.7, 11.8, 122.5, 126.7, 129.4, 129.8, 130.7, 135.1, 38.6, 141.3, 142.8, 150.1, 176.9.

**Synthesis of the Hexafluorophosphate Salt of 28**

A solution of 18 (300 mg, 1 mmol) and the iodide salt of 24 (300 mg, 1 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 28 (184 mg, 32%) as a purple solid. ESIMS: $m/z = 429$ [M]$^+$ ($m/z$ calcd. for C$_{31}$H$_{29}$N$_2$ = 429); $^1$H NMR (CDCl$_3$): $\delta = 1.82$ (6H, s), 4.35 (3H, s), 2.45 (3H, s), 7.02 (2H, d, 9 Hz), 7.20 (2H, d, 8 Hz), 7.24 (2H, t, 7 Hz), 7.38 (5H, t, 8 Hz), 7.46–7.56 (5H, m), 7.61 (1H, d, 16 Hz), 8.02 (2H, d, 9 Hz), 8.08 (1H, d, 16 Hz); $^{13}$C NMR (CDCl$_3$): $\delta = 27.3, 33.9, 52.2, 107.5, 114.1, 119.7, 122.8, 126.5, 126.7, 127.0, 129.2, 129.8, 130.3, 133.6, 142.0, 142.9, 145.6, 154.2, 154.7, 181.1.
Synthesis of the Hexafluorophosphate Salt of 29

A solution of 19 (77 µL, 0.8 mmol) and the iodide salt of 24 (250 mg, 0.8 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 29 (80 mg, 24%) as a brown solid. FABMS: m/z = 268 [M – PF$_6$]$^+$ (m/z calcd. for C$_{17}$H$_{18}$NS = 268); $^1$H NMR (CDCl$_3$): δ = 1.87 (6H, s), 4.39 (3H, s), 7.29 (1H, s), 7.40 (1H, d, 16 Hz), 7.55–7.61 (4H, m), 7.82 (1H, d, 5 Hz), 8.34 (1H, s), 8.51 (1H, d, 16 Hz).

Synthesis of the Hexafluorophosphate Salt of 30

A solution of 20 (161 mg, 0.8 mmol) and the iodide salt of 24 (250 mg, 0.8 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 30 (100 mg, 25%) as a brown solid. FABMS: m/z = 351 [M – PF$_6$]$^+$ (m/z calcd. for C$_{21}$H$_{20}$NS$_2$ = 350); $^1$H NMR (CDCl$_3$): δ = 1.86 (6H, s), 4.32 (3H, s), 7.10–7.13 (1H, m), 7.20–7.24 (1H, m), 7.34 (1H, d, 4 Hz), 7.42–7.46 (2H, m), 7.53–7.56 (4H, m), 8.37 (1H, d, 4 Hz), 8.53 (1H, d, 15 Hz).
Synthesis of the Hexafluorophosphate Salt of 31

A solution of 21 (116 mg, 1.0 mmol) and the iodide salt of 24 (180 mg, 1.0 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 31 (100 mg, 36%) as a brown solid. ESIMS: $m/z$ = 268 [M]$^+$ ($m/z$ calcd. for C$_{17}$H$_{18}$NS = 268); $^1$H NMR (CDCl$_3$): $\delta$ = 1.79 (6H, s), 4.35 (3H, s), 7.47–7.63 (6H, m), 7.88 (1H, s), 8.23 (1H, d, 17 Hz), 8.36 (1H, s); $^{13}$C NMR [(CD$_3$)$_2$CO]: $\delta$ = 25.2, 34.1, 52.6, 112.4, 114.9, 122.8, 125.8, 128.6, 129.3, 129.6, 136.5, 138.8, 142.2, 143.6, 147.4, 183.0.

Synthesis of the Hexafluorophosphate Salt of 33

A solution of 4-methoxybenzaldehyde (68 mg, 0.5 mmol) and the iodide salt of 24 (100 mg, 0.3 mmol) in EtOH (20 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 33 (120 mg, 83%) as a yellow-orange solid. ESIMS: $m/z$ = 292 [M – PF$_6$]$^+$ ($m/z$ calcd. for C$_{20}$H$_{22}$NO = 292); $^1$H NMR [(CD$_3$)$_2$CO]: $\delta$ = 1.85 (6H, s), 3.92 (3H, s), 4.45 (3H, s), 7.07 (2H, d, 8 Hz), 7.54 (2H, s), 7.57 (2H, s), 7.78 (1H, d, 16 Hz), 8.18 (1H, d, 16 Hz), 8.23 (2H, d, 8 Hz); $^{13}$C NMR
(CDCl₃): δ = 25.8, 34.2, 52.8, 55.8, 110.5, 115.1, 115.5, 115.7, 123.2, 127.8, 129.6,
129.7, 133.4, 142.6, 143.9, 154.5, 165.1, 182.8.

Synthesis of the Hexafluorophosphate Salt of 34

A solution of 4-hydroxybenzaldehyde (61 mg, 0.5 mmol) and the iodide salt of 24 (100
mg, 0.3 mmol) in EtOH (20 mL) was heated under reflux for 24 hours. After cooling
down to ambient temperature, the solvent was distilled off under reduced pressure and the
residue was dissolved in CH₂Cl₂ (5 mL). The addition of Et₂O (30 mL) caused the
precipitation of a solid, which was filtered off and dissolved in Me₂CO (5 mL). After the
addition of a saturated aqueous solution of NH₄PF₆ (5 mL), the solution was concentrated
under reduced pressure to half of its original volume and the resulting precipitate was
filtered off to give the hexafluorophosphate salt of 34 (110 mg, 78%) as a yellowish-
orange solid. ESIMS: m/z = 278 [M – PF₆]⁺ (m/z calcld. for C₁₉H₂₀NO = 278); ¹H NMR
[(CD₃)₂CO]: δ = 1.85 (6H, s), 4.12 (3H, s), 6.98 (2H, d, 9 Hz), 7.44 (1H, d, 9 Hz), 7.61–
7.66 (2H, m), 7.43–7.81 (2H, m), 8.00 (2H, d, 9 Hz), 8.39 (1H, d, 16 Hz); ¹³C NMR
[(CD₃)₂CO]: δ = 25.9, 34.1, 52.7, 109.7, 114.9, 117.0, 123.1, 126.9, 129.6, 133.8, 142.6,
143.8, 154.9, 163.7, 182.7, 205.5.

Synthesis of 37a

A solution of 16 (310 mg, 1 mmol), 42 (194 mg, 1 mmol) and TFA (0.5 mL, 6.5 mmol)
in EtOH (10 mL) was heated under reflux for 3 h. After cooling down to ambient
temperature, the solvent was distilled off under reduced pressure and the residue was
dissolved in CH₂Cl₂ (5 mL). The addition of Et₂O (50 mL) caused the precipitation of a
solid, which was filtered off, dissolved in CH₂Cl₂ (20 mL) and washed with H₂O (20
mL). The organic phase was dried over Na₂SO₄ and the solvent was distilled off under
reduced pressure to give 37a (306 mg, 63 %) as an orange solid. FABMS: m/z= 488 [M+
H]⁺; HRESIMS: m/z = 487.2009 [M + H]⁺ (m/z calcld. for C₃₂H₂₇N₂O₃ = 487.2016); ¹H
NMR (CDCl$_3$): $\delta$ = 1.28 (6H, bs), 3.91 (2H, s), 4.62 (2H, s), 6.41 (1H, d, 16 Hz), 6.65 (1H, d, 8 Hz), 6.87–6.92 (3H, m), 7.10–7.17 (2H, m), 7.33 (1H, t, 7 Hz), 7.39 (1H, d, 7 Hz), 7.43 (1H, d, 9 Hz), 7.56 (1H, d, 7 Hz), 7.62 (1H, s), 7.74 (1H, d, 8 Hz), 7.79 (1H, d, 8 Hz), 7.99-8.03 (2H, m); $^{13}$C NMR (CDCl$_3$): $\delta$ = 14.6, 23.1, 32.0, 37.2, 50.6, 104.3, 109.2, 118.1, 120.5, 121.1, 122.8, 123.6, 123.7, 124.4, 125.5, 127.3, 127.5, 128.1, 134.5, 136.9, 138.6, 141.0, 141.5, 142.7, 144.0, 144.2, 146.9, 159.7.

**Synthesis of 38a**

A solution of 16 (310 mg, 1 mmol), 43 (230 mg, 1 mmol) and TFA (0.5 mL, 6.5 mmol) in EtOH (10 mL) was heated under reflux for 24 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (50 mL) caused the precipitation of a solid, which was filtered off, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distill off under reduced pressure to give 38a (199 mg, 38 %) as a purple solid. ESIMS: $m/z$ = 524 [M + H]$^+$; HRESIMS: $m/z$ = 523.2020 [M + H]$^+$ ($m/z$ calcd. for C$_{35}$H$_{27}$N$_2$O$_3$ = 523.2016); $^1$H NMR (CDCl$_3$): $\delta$ = 1.57 (6H, bs), 4.71(2H, s), 6.60 (1H, d, 16 Hz), 6.70 (1H, d, 8 Hz), 6.95 (1H, t, 8 Hz), 7.02 (1H, d, 9 Hz), 7.17–7.24 (2H, m), 7.95 (1H, d, 16 Hz), 8.02–8.21 (11H, m); $^{13}$C NMR (CDCl$_3$): $\delta$ = 30.1, 41.5, 50.6, 109.4, 118.2, 120.5, 122.8, 122.9, 123.8, 124.5, 124.7, 125.1, 125.3, 125.4, 125.9, 126.2, 126.6, 127.8, 128.2, 128.7, 131.1, 131.8, 138.7, 141.1, 146.8, 159.9.

**Synthesis of 40a**

A solution of 16 (231 mg, 0.7 mmol), 45 (150 mg, 0.7 mmol), and TFA (55 µL, 0.7 mmol) in EtOH (10 mL) was heated under reflux for 24 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (50 mL) caused the precipitation
of a solid, which was filtered off, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure to give 40a (110 mg, 30 %) as a red solid. FABMS: $m/z$ = 495 [M + H]$^+$. HRESIMS: $m/z$ = 494.2430 [M + H]$^+$ (m/z calcd. for C$_{31}$H$_{32}$N$_3$O$_3$ = 494.2438); $^1$H NMR (CDCl$_3$): $\delta$ = 1.68 (6H, s), 1.74 (6H, s), 3.72 (3H, s), 5.29 (2H, s), 6.47 (1H, d, 13 Hz), 7.15 (1H, d, 7 Hz), 7.30–7.51 (9H, m), 7.79 (1H, d, 2 Hz), 8.08 (1H, dd, 3 and 9 Hz), 8.33 (1H, t, 13 Hz).

**Synthesis of the Hexafluorophosphate Salt of 47**

A solution of 42 (210 mg, 1 mmol) and the iodide salt of 24 (300 mg, 1 mmol) in EtOH (10 mL) was heated under reflux for 24 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 47 (322 mg, 65 %) as an orange solid. FABMS: $m/z$ = 351 [M – PF$_6$]$^+$; $^1$H NMR (CDCl$_3$): $\delta$ = 1.88 (6H, s), 4.09 (2H, s), 4.48 (3H, s), 7.42–7.44 (2H, m), 7.56–7.60 (5H, m), 7.86–7.96 (3H, m), 8.06 (1H, d, 8 Hz), 8.29 (1H, d, 16 Hz), 8.66 (1H, s); $^{13}$C NMR (CDCl$_3$): $\delta$ = 15.7, 23.5, 27.5, 37.3, 37.6, 52.7, 66.2, 112.4, 115.2, 121.1, 121.7, 122.9, 123.4, 125.9, 127.6, 128.0, 129.2, 130.2, 130.5, 132.3, 132.7, 141.9, 143.2, 155.7, 183.6.

**Synthesis of the Hexafluorophosphate Salt of 48**

A solution of 43 (200 mg, 0.9 mmol) and the iodide salt of 24 (240 mg, 0.8 mmol) in EtOH (10 mL) was heated under reflux for 24 h. After cooling down to ambient
temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 48 (310 mg, 73 %) as a purple solid. ESIMS: $m/z = 386 \ [M - PF_6]^+$; $^1$H NMR (CDCl$_3$): $\delta = 2.04$ (6H, s), 4.64 (3H, s), 7.59–7.68 (4H, m), 8.07–8.14 (2H, m), 8.24 (1H, d, 9 Hz), 8.29–8.33 (3H, m), 8.38 (2H, t, 8 Hz), 8.48 (1H, d, 9 Hz), 9.33 (1H, d, 16 Hz), 9.44 (1H, d, 9 Hz); $^{13}$C NMR (CDCl$_3$): $\delta = 26.6, 35.8, 53.2, 115.7, 116.2, 123.3, 123.8, 124.2, 124.6, 126.6, 127.9, 128.2, 128.3, 128.4, 130.0, 130.3, 130.8, 130.9, 131.2, 131.6, 131.7, 135.2, 142.9, 144.4, 148.6, 182.4.

**Synthesis of the Hexafluorophosphate Salt of 50**

A solution of 45 (500 mg, 1.7 mmol) and the iodide salt of 24 (334 mg, 1.7 mmol) in EtOH (10 mL) was heated under reflux for 24 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (5 mL). The addition of Et$_2$O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 50 (480 mg, 80 %) as a purple solid. ESIMS: $m/z = 357 \ [M - PF_6]^+$; $^1$H NMR (CDCl$_3$): $\delta = 1.72$ (12H, s), 3.82 (6H, s), 7.09 (2H, d, 16 Hz), 7.26-7.28 (2H, m), 7.35-7.43 (6H, m), 8.44 (1H, t, 13 Hz), 8.08 (1H, d, 9 Hz), 8.57 (1H, d, 16 Hz), 9.97 (1H, s).
Synthesis of 52a

A solution of 58 (480 mg, 1.4 mmol) and 3-ethyl-2,4-dimethylpyrrole (330 mg, 2.7 mmol) and TFA (10 µL, 0.1 mmol) in CH₂Cl₂ (100 mL) was stirred for 3 h at ambient temperature under Ar. After the addition of a solution of DDQ (310 mg, 1.4 mmol) in CH₂Cl₂ (15 mL), the mixture was stirred for a further 30 min. Then, Et₃N (3 mL, 21 mmol) and BF₃·Et₂O (3 mL, 24 mmol) were added and the mixture was stirred for a further 30 min, washed with H₂O (3 × 100 mL) and dried over Na₂SO₄. The solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO₂: hexanes/EtOAc (1:1, v/v)] to yield 52a (187 mg, 22%) as an orange powder. ESIMS: m/z = 628.3351 [M]⁺ (m/z calcd. for C₄₀H₄₂BF₂N₃O = 628.3312); ¹H NMR (CDCl₃): δ = 1.72 (1H, s), 4.73 (2H, s), 6.46 (1H, s), 7.02 (1H, d, 6 Hz), 7.82 (1H, dd, 3 and 6 Hz), 7.90 (1H, d, 3 Hz), 9.90 (1H, s); ¹³C NMR (CDCl₃): δ = 11.4, 12.6, 12.9, 15.0, 15.1, 17.4, 17.5, 19.1, 28.3, 41.9, 49.7, 104.4, 109.2, 119.2, 120.5, 121.9, 122.7, 126.5, 127.7, 128.0, 128.1, 128.7, 131.3, 131.5, 133.0, 136.8, 138.2, 138.8, 140.4, 148.2, 153.8, 153.9, 154.7.

Synthesis of 53a

A solution of 3-ethyl-2,4-dimethylpyrrole (54 mg, 0.4 mmol), 61 (85 mg, 0.2 mmol) and TFA (10 µL, 0.1 mmol) in CH₂Cl₂ (100 mL) was stirred for 12 h at ambient temperature under Ar. After the addition of a solution of TCBQ (52 mg, 0.2 mmol) in CH₂Cl₂ (15 mL), the mixture was stirred for a further 30 min. Then, Et₃N (2 mL, 14 mmol) and BF₃·Et₂O (2 mL, 16 mmol) were added and the mixture was stirred for a further 30 min, washed with H₂O (3 × 100 mL) and dried over Na₂SO₄. The solvent was distilled off under reduced pressure and the residue was purified
by column chromatography [SiO$_2$: hexanes/EtOAc (1:1, v/v)] to yield 53a (81 mg, 57%) as an orange powder. ESIMS: $m/z = 697.3182$ [M + Na]$^+$ ($m/z$ calcd. for C$_{40}$H$_{41}$BF$_2$N$_4$NaO$_3$ = 697.3139); $^1$H NMR (CDCl$_3$): $\delta = 0.90$ (3H, s), 0.94–0.97 (9H, m), 1.34 (3H, s), 1.64 (3H, s), 2.18–2.37 (4H, m), 2.54 (6H, s), 4.54 (1H, d, 18 Hz), 4.69 (1H, d, 18 Hz), 6.76 (1H, d, 8 Hz), 6.94 (2H, t, 7 Hz), 7.20 (2H, t, 7 Hz), 7.36 (2H, d, 7 Hz), 7.71–7.79 (2H, m), 7.96–7.99 (2H, m); $^{13}$C NMR (CDCl$_3$): $\delta = 12.9$, 15.0, 17.4, 19.0, 28.2, 39.1, 41.3, 49.9, 105.5, 109.5, 118.7, 120.5, 121.5, 123.0, 123.2, 124.3, 128.3, 129.1, 131.0, 133.4, 137.0, 137.1, 137.6, 138.3, 139.5, 141.4, 147.0, 159.3.

**Synthesis of 54a**

A mixture of 61 (68 mg, 0.2 mmol), 62 (65 mg, 0.2 mmol), piperidine (0.3 mL, 3 mmol) and acetic acid (0.2 mL, 3 mmol) in benzene (20 mL) was heated for 12 h under reflux in a Dean-Stark apparatus. After cooling down to ambient temperature, the solvent distilled off under reduced pressure and the residue was purified by column chromatography [SiO$_2$: hexanes/EtOAc (1:1, v/v)] to afford 54a (25 mg, 19%) as a purple solid. ESIMS: $m/z = 785.3487$ [M + Na]$^+$ ($m/z$ calcd. for C$_{47}$H$_{45}$BF$_2$N$_4$NaO$_3$ = 785.3453); $^1$H NMR (CDCl$_3$): $\delta = 0.90$ (3H, s), 1.00 (3H, t, 7 Hz), 1.14 (3H, t, 7 Hz), 1.31 (3H, s), 1.32 (3H, s), 1.61 (3H, s), 2.33 (2H, q, 7 Hz), 2.56–2.58 (5H, m), 4.56 (1H, d, 18 Hz), 4.66 (1H, d, 18 Hz), 6.74 (1H, d, 8 Hz), 6.92 (2H, t, 7 Hz), 7.14–7.19 (3H, m), 7.30–7.31 (2H, m), 7.49–7.51 (3H, m), 7.63 (4H, bs), 7.74 (1H, d, 17 Hz), 7.94–7.97 (2H, m); $^{13}$C NMR (CDCl$_3$): $\delta = 13.3$, 17.5, 18.7, 18.9, 50.2, 75.4, 105.6, 120.5, 121.5, 123.6, 124.1, 128.1, 128.8, 129.4, 132.2, 132.7, 133.3, 134.5, 136.1, 138.0, 138.7, 140.0, 140.2, 141.3, 147.2, 157.0, 159.5.
Synthesis of 55a

A mixture of 16 (183 mg, 0.6 mmol), 63 (104 mg, 0.3 mmol) and TFA (0.3 mmol, 20 µL) in EtOH (20 mL) was heated for 12 h under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO2: hexanes/EtOAc (4:1, v/v)] to afford 55a (20 mg, 10%) as a purple solid. ESIMS: m/z = 645.2894 [M + H]+ (m/z calcd. for C38H36BF2N4O3 = 645.2850); 1H NMR (CDCl3): δ = 1.26 (6H, s), 1.31 (3H, s), 1.38 (3H, s), 2.57 (3H, s), 2.59 (3H, s), 4.59 (2H, s), 5.89 (1H, d, 16 Hz), 6.03 (1H, s), 6.65 (2H, d, 8 Hz), 6.82–6.93 (2H, m), 7.10–7.15 (2H, m), 7.24–7.26 (1H, m), 7.48–7.50 (3H, m), 7.96–8.06 (2H, m); 13C NMR (CDCl3): δ = 13.1, 14.2, 15.0, 15.2, 30.1, 41.3, 50.1, 76.5, 76.8, 77.6, 109.3, 118.1, 120.4, 121.4, 122.6, 122.8, 123.6, 124.1, 124.5, 125.3, 125.8, 126.9, 128.1, 128.3, 129.6, 129.7, 131.1, 132.6, 135.2, 138.5, 138.9, 140.9, 142.4, 145.0, 146.6, 153.7.

Synthesis of 56a

A solution of 65 (100 mg, 0.2 mmol) and 2-chloromethyl-4-nitrophenol (37 mg, 0.2 mmol) in MeCN (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH2Cl2 (3 mL). The addition of Et2O (20 mL) caused the precipitation of a purple solid. The solid was dissolved in CH2Cl2 (30 mL) and washed with H2O (20 mL). The organic phase was dried over Na2SO4 and the solvent was distilled off under reduced pressure to give 56a (87 mg, 70%) as a red solid. ESIMS: m/z = 771.3734 [M + H]+ (m/z calcd. for C48H47BN4O5 = 771.3720); 1H NMR (CDCl3): δ = 0.93 (6H, t, 7 Hz), 1.29 (6H, s), 1.51 (6H, bs), 2.02 (3H, s), 2.03 (3H, s), 2.22 (4H, q, 8 Hz), 4.81 (2H, bs),
6.74–6.77 (1H, m), 7.02 (4H, bs), 7.21–7.28 (5H, m), 7.50–7.52 (4H, m), 7.98–8.01 (3H, m).

**Synthesis of 58**

A solution of 57 (511 mg, 3 mmol) and 26 (700 mg, 3 mmol) in MeCN (20 mL) was heated for 24 h under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (20 mL). The resulting solution was washed with H$_2$O (2 × 20 mL). The solvent of the organic phase was distilled off under reduced pressure and the residue was purified by column chromatography (SiO$_2$: CH$_2$Cl$_2$) to afford 58 (480 mg, 45%) as a white solid. ESIMS: $m/z$ = 356.1641 [M + H]$^+$ (m/z calcd. for C$_{24}$H$_{22}$NO$_2$ = 356.1645); $^1$H NMR (CDCl$_3$): $\delta$ = 0.84 (3H, s), 1.60 (3H, s), 4.54 (1H, d, 8 Hz), 4.64 (1H, d, 8 Hz), 6.73 (1H, 8 Hz), 6.91 (2H, q, 8 Hz), 7.11–7.18 (2 H, m), 7.83 (3H, m), 7.53 (1H, s), 7.56 (1H, dd, 2 and 8 Hz), 7.66 (2H, d, 8 Hz). 9.72 (1H, s); $^{13}$C NMR (CDCl$_3$): $\delta$ = 18.2, 19.9, 41.2, 50.2, 105.2, 108.5, 110.8, 117.6, 119.7, 120.1, 120.7,122.2, 127.5, 128.4, 129.7, 130.0, 130.5, 136.7, 138.3, 147.8, 159.4, 192.3.

**Synthesis of 59**

A solution of i-propyltolylketone (2.88 g, 18 mmol) and phenylhydrazine (1.78 mL, 18 mmol) in acetic acid (12 mL) was heated for 24 h under reflux. After cooling down to ambient temperature, the solution was diluted with H$_2$O (20 mL) and the pH was adjusted to ca. 8 with aqueous KOH (0.3 M). Then, the mixture was extracted with CH$_2$Cl$_2$ (3 × 20 mL). The organic phase was dried over Na$_2$SO$_4$, filtered and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO$_2$: hexanes/CH$_2$Cl$_2$ (1:2, v/v)] to afford 59 (2.8 g, 68%) as a white solid. ESIMS: $m/z$ =
236.1442 [M + H]$^+$ (m/z calcd. for C₁₇H₁₈N = 236.1434); $^1$H NMR (CDCl₃): δ = 1.58 (6H, s), 2.40 (3H, s), 7.26–7.36 (4H, m), 7.38 (2H, dt, 1 and 8 Hz), 7.39 (1H, d, 8 Hz), 8.14 (2H, d, 8 Hz); $^{13}$C NMR (CDCl₃): δ = 21.9, 24.6, 121.4, 128.2, 129.9, 131.9, 141.3, 148.1, 153.7, 183.5.

**Synthesis of 60**

A suspension of 59 (80 mg, 0.3 mmol), NBS (121 mg, 0.7 mmol) and AIBN (16 mg, 0.1 mmol) in CCl₄ (10 mL) was heated for 23 h under reflux and Ar. The mixture was diluted with EtOAc (25 mL), extracted with aqueous HCl (3%, 3 × 20 mL), washed with brine and dried over Na₂SO₄. The organic phase was filtered and the solvent was distilled off under reduced pressure. The residue was dissolved in MeCN (0.6 mL) and diluted with a solution of AgNO₃ (340 mg, 2 mmol) in H₂O (0.3 mL). The mixture was heated for 20 min under reflux, allowed to cool down to ambient temperature, filtered and washed with CH₂Cl₂ (3 × 5 mL). The organic phase was washed with H₂O (25 mL) and dried over Na₂SO₄. The solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO₂: hexanes/CH₂Cl₂ (1:3, v/v)] to afford 60 (60 mg, 80%) as a yellow gel. FABMS: m/z = 250 [M + H]$^+$; $^1$H NMR (CDCl₃): δ = 1.58 (6H, s), 7.22–7.38 (3H, m), 7.72 (1H, d, 8 Hz), 7.97 (4H, d, 8 Hz), 8.41 (4H, d, 8 Hz), 10.06 (1H, s); $^{13}$C NMR (CDCl₃): δ = 24.8, 54.0, 121.5, 121.8, 127.1, 128.4, 128.9, 129.1, 130.2, 137.6, 138.9, 148.1, 153.2, 182.2, 192.1.

**Synthesis of 61**

A solution of 60 (130 mg, 0.5 mmol) and 2-chloromethyl-4-nitrophenol (107 mg, 0.6 mmol) in MeCN (30 mL) was heated for 48 h under reflux and Ar. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the
residue was dissolved in CH₂Cl₂ (15 mL) and washed with H₂O (20 mL). The solvent of the organic phase was distilled off under reduced pressure and the residue was purified by column chromatography (SiO₂: CH₂Cl₂) to afford 61 (116 mg, 56%) as a white solid. ESIMS: m/z = 401 [M + H]⁺; ¹H NMR (CDCl₃): δ = 0.85 (3H, s), 1.61 (3H, s), 4.50 (1H, d, 18 Hz), 4.68 (1H, d, 18 Hz), 6.75 (1H, d, 8 Hz), 6.94 (2H, t, 8 Hz), 7.17–7.21 (2H, m), 7.85 (2H, bs), 7.94–7.97 (4H, m); ¹³C NMR (CDCl₃): δ = 41.1, 50.8, 105.2, 117.4, 119.6, 120.4, 121.9, 122.6, 123.1, 124.0, 125.3, 127.2, 128.1, 129.5, 130.3, 131.4, 137.0, 137.6, 141.4, 143.2, 147.0, 159.0.

**Synthesis of 63**

A solution of 68 (200 mg, 0.6 mmol) in 1,2-dichloroethane (50 mL) was added to a solution of POCl₃ (2 mL, 22 mmol) in dry N,N'-dimethylformamide (2 mL) maintained at ambient temperature under Ar. The mixture was heated at 50 °C for 2 hours and, after cooling down to ambient temperature, was slowly poured into a saturated aqueous solution of NaHCO₃ (200 mL) maintained in an ice bath. The resulting mixture was stirred for a further 30 min and washed with H₂O (50 mL). The organic layer was dried over Na₂SO₄ and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO₂: hexanes/EtOAc (9:1, v/v)] to afford 63 (100 mg, 33%) as an orange solid. ESIMS: m/z = 375 [M + Na]⁺; ¹H NMR (CDCl₃): δ = 1.44 (3H, s), 1.67 (3H, s), 2.63 (3H, s), 2.84 (3H, s), 6.17 (1H, s), 7.21–7.31 (2H, m), 7.53–7.57 (3H, m), 9.96 (1H, s); ¹³C NMR (CDCl₃): δ = 11.6, 13.0, 14.8, 15.1, 97.6, 99.9, 101.4, 102.4, 124.1, 127.7, 129.5, 134.1, 143.6, 147.4, 156.5, 161.7, 186.0.
**Synthesis of 64**

A solution of 2,3,3-trimethyl-3H-indole (480 mg, 2 mmol), 3,4-dihydroxybenzaldehyde (330 mg, 2.4 mmol) and HBr (0.5 mL, 33 % in AcOH) in EtOH (10 mL) was heated under reflux for 2 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$ (30 mL) and washed with a saturated aqueous solution of NaHCO$_3$ (2 × 20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO$_2$: hexane/EtOAc (1:1, v/v)] to afford 64 (120 mg, 21%) as a red solid. ESI-MS: $m/z = 280$ [M + H]$^+$; $^1$H NMR (CDCl$_3$): $\delta = 1.45$ (6H, s), 6.8 (1H, s), 6.84–6.87 (2H, m), 7.18 (1H, s), 7.23–7.27 (1H, m), 7.32 (2H, t, 8 Hz), 7.52–7.59 (2H, m).

**Synthesis of 65**

AlCl$_3$ (58 mg, 0.4 mmol) was added to a solution of 62 (110 mg, 0.3 mmol) in dry CH$_2$Cl$_2$ (10 mL) maintained under argon. The suspension was stirred for 15 min and then 64 (120 mg, 0.43 mmol) was added. The mixture was stirred for a further 20 min and then washed with a saturated aqueous solution of NaHCO$_3$ (2 × 20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO$_2$: CH$_2$Cl$_2$] to give 65 (155 mg, 86%) as a red solid. ESI-MS: $m/z = 620.3430$ [M + H]$^+$ (m/z calcd. for C$_{41}$H$_{43}$BN$_3$O$_2$ = 620.3450); $^1$H NMR (CDCl$_3$): $\delta = 0.95$ (6H, t, 7 Hz), 1.30 (6H, s), 1.50 (3H, s), 1.60 (3H, s), 2.08 (6H, s), 2.24 (4H, q, 7 Hz), 6.79–6.82 (1H, m), 6.95 (1H, d, 18 Hz), 7.09 (1H, d, 9 Hz), 7.16 (1H, s), 7.22–7.33 (6H, m), 7.46–7.52 (2H, m), 7.63 (1H, d, 9 Hz), 7.73 (1H, d, 18 Hz).
Synthesis of 67

A mixture of benzaldehyde (16 mg, 0.16 mmol), 62 (61 mg, 0.16 mmol), piperidine (0.3 mL, 3 mmol) and acetic acid (0.2 mL, 3 mmol) in benzene (20 mL) was heated for 12 h under reflux in a Dean-Stark apparatus. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO₂: hexanes/EtOAc (1:1, v/v)] to afford 67 (15 mg, 20%) as a purple solid. ESIMS: m/z = 491 [M + Na]⁺; ¹H NMR (CDCl₃): δ = 0.99 (3H, t, 8 Hz), 1.15 (3H, t, 8 Hz), 1.29 (3H, s), 1.31 (3H, s), 1.57 (3H, s), 2.60 (4H, q, 8 Hz), 7.19 (1H, d, 16 Hz), 7.28–7.40 (5H, m), 7.48–7.50 (3H, m), 7.60 (2H, d, 8 Hz), 7.73 (1H, d, 16 Hz).

Synthesis of 69

A mixture of catechol (25 mg, 0.23 mmol), 62 (57 mg, 0.15 mmol) and AlCl₃ (30 mg, 0.23 mmol) in dry CH₂Cl₂ (6 mL) was stirred for 30 min under Ar at ambient temperature. After washing with H₂O (50 mL), the solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO₂: CH₂Cl₂/hexanes (1:1, v/v)] to afford 69 (61 mg, 95%) as a dark red solid. ESI MS: m/z = 451 [M + H]⁺; ¹H NMR (CDCl₃): δ = 0.93 (6H, t, 8 Hz), 1.29 (6H, s), 2.06 (6H, s), 2.25 (4H, q, 8 Hz), 6.80 (4H, s), 7.30–7.32 (2H, m), 7.48–7.50 (3H, m); ¹³C NMR (CDCl₃): δ = 12.2, 13.1, 15.0, 17.5, 109.5, 119.8, 128.7, 129.1, 129.5, 131.9, 133.8, 136.4, 139.6, 152.4, 155.8.

Synthesis of the Hexafluorophosphate Salt of 71

A solution of 75 (340 mg, 0.7 mmol) and methyl iodide (1.0 mL, 16 mmol) in MeCN (20 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in
CH$_2$Cl$_2$ (3 mL). The addition of Et$_2$O (20 mL) caused the precipitation of a purple solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 71 (324 mg, 73%) as a dark red solid. ESI-MS: $m/z = 538.3217$ [M – PF$_6$]$^+$ ($m/z$ calcd. for C$_{34}$H$_{39}$BF$_2$N$_3$ = 538.3206); $^1$H NMR (CDCl$_3$): $\delta$ = 0.98 (6H, t, 8 Hz), 1.30 (6H, s), 1.70 (6H, s), 2.30 (4H, q, 8 Hz), 2.52 (6H, s), 4.20 (3H, s), 7.56–7.63 (4H, m), 7.80 (2H, d, 8 Hz), 8.13 (2H, d, 8 Hz); $^{13}$C NMR (CDCl$_3$): $\delta$ = 12.3, 13.0, 15.0, 17.5, 23.2, 39.1, 56.1, 117.4, 123.5, 126.0, 130.0, 130.2, 130.3, 130.6, 131.4, 134.0, 137.6, 138.0, 141.4, 142.0, 142.3, 155.2, 190.7.

**Synthesis of the Hexafluorophosphate Salt of 72**

A solution of 76 (34 mg, 0.06 mmol) and methyl iodide (1.0 mL, 16 mmol) in MeCN (20 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (3 mL). The addition of Et$_2$O (20 mL) caused the precipitation of a purple solid, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 72 (28 mg, 65%) as a purple solid. ESI-MS: $m/z = 626$ [M – PF$_6$]$^+$; $^1$H NMR (CD$_3$CN): $\delta$ =1.01 (3H, t, 8 Hz), 1.17 (3H, t, 8 Hz), 1.36 (3H, s), 1.37 (3H, s), 1.66 (6H, s), 2.37 (2H, q, 8 Hz), 2.56 (3H, s), 2.68 (2H, q, 8 Hz), 3.96 (3H, s), 7.28–7.30 (3H, m), 7.58–7.60 (3H, m), 7.64–7.76 (4H, m), 7.81 (1H, d, 8 Hz), 7.82–7.85 (2H, m), 7.94 (2H, d, 8 Hz).
Synthesis of the Hexafluorophosphate Salt of 73

A mixture of 63 (50 mg, 0.14 mmol) and the iodide salt of 24 (43 mg, 0.14 mmol) in EtOH (20 mL) was heated for 24 hours under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH₂Cl₂ (5 mL). The addition of Et₂O (30 mL) caused the precipitation of a solid, which was filtered off and dissolved in Me₂CO (5 mL). After the addition of a saturated aqueous solution of NH₄PF₆ (5 mL), the solution was concentrated under reduced pressure to half of its original volume and the resulting precipitate was filtered off to give the hexafluorophosphate salt of 73 (25 mg, 36%) as a purple solid. ESIMS: m/z = 508.2751 [M – PF₆]⁺ (m/z calcd. for C₃₂H₃₃BF₂N₃ = 508.2736); ¹H NMR [(CD₃)₂CO]: δ = 1.51 (3H, s), 1.73 (3H, s), 1.88 (6H, s), 2.65 (3H, s), 4.20 (3H, s), 6.48 (1H, s), 7.09 (1H, d, 8 Hz), 7.53–7.56 (2H, m), 7.62–7.69 (5H, m), 7.76–7.85 (2H, m), 8.32 (1H, d, 8 Hz); ¹³C NMR [(CD₃)₂CO]: δ = 12.5, 13.5, 14.3, 25.9, 33.6, 51.9, 97.6, 98.6, 99.9, 101.4, 110.3, 114.4, 122.8, 128.8, 129.0, 129.2, 129.7, 129.9, 134.0, 143.1, 145.9, 148.4.

Synthesis of the Iodide Salt of 74

A solution of 65 (55 mg, 0.1 mmol) and methyl iodide (0.5 mL, 8 mmol) in MeCN (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH₂Cl₂ (3 mL). The addition of Et₂O (20 mL) caused the precipitation of a purple solid, which was filtered off to give the iodide salt of 74 (45 mg, 66%) as red solid. ESIMS: m/z = 634.3624 [M – I]⁺ (m/z calcd. for C₄₂H₄₅BN₃O₂ = 634.3607); ¹H NMR (CD₃CN): δ = 0.93 (6H, t, 8 Hz), 1.26 (6H, s), 1.83 (6H, s), 1.98 (6H, s), 2.25 (4H, q, 8 Hz), 4.26 (3H, s), 6.91 (1H, d, 8 Hz), 6.98 (1H, d, 8 Hz), 7.41–7.66 (11H, m), 8.16 (1H, d, 16 Hz); ¹³C
NMR (CDCl$_3$): $\delta = 11.9, 12.7, 14.7, 17.0, 27.4, 29.7, 35.7, 51.7, 107.9, 108.5, 109.8, 114.0, 119.3, 122.7, 127.2, 127.9, 128.0, 128.9, 129.1, 129.2, 129.3, 131.4, 131.5, 134.0, 140.2, 140.5, 141.7, 153.4, 155.4, 156.3, 160.7, 180.9.

**Synthesis of 75**

A solution of 3-ethyl-2,4-dimethylpyrrole (370 mg, 3 mmol), **60** (370 mg, 1.5 mmol) and TFA (10 µL, 0.1 mmol) in CH$_2$Cl$_2$ (300 mL) was stirred for 12 hours at ambient temperature under Ar. After the addition of a solution of TCBQ (370 mg, 1.5 mmol) in CH$_2$Cl$_2$ (30 mL), the mixture was stirred for a further 30 min. Then, Et$_3$N (5 mL, 35 mmol) and BF$_3$·Et$_2$O (5 mL, 40 mmol) were added and the mixture was stirred for a further 30 min, washed with H$_2$O (3 × 100 mL) and dried over Na$_2$SO$_4$. The solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO$_2$: hexanes/EtOAc (2:1, v/v)] to yield **75** (370 mg, 48%) as an orange powder. ESIMS: $m/z = 524.3066$ [M + H]$^+$ ($m/z$ calcd. for C$_{33}$H$_{37}$BF$_2$N$_3 = 524.3049$); $^1$H NMR (CD$_3$CN): $\delta = 1.00$ (6H, t, 8 Hz), 1.36 (6H, s), 1.64 (6H, s), 2.31 (4H, q, 8 Hz), 2.56 (6H, s), 7.32 (1H, d, 8 Hz), 7.39 (2H, d, 8 Hz), 7.44 (2H, d, 8 Hz), 7.23 (1H, d, 8 Hz), 7.26 (2H, d, 8 Hz); $^{13}$C NMR (CDCl$_3$): $\delta = 11.2, 12.7, 14.7, 17.2, 24.7, 24.8, 53.8, 121.1, 121.2, 126.4, 128.0, 128.9, 129.0, 130.6, 133.1, 134.0, 138.2, 138.4, 139.3, 147.6, 153.0, 154.2, 182.9.

**Synthesis of 76**

A solution of **60** (250 mg, 1 mmol), **62** (430 mg, 1 mmol), piperidine (1 mL, 10 mmol) and acetic acid (0.8 mL, 10 mmol) in benzene (30 mL) was heated under reflux for 12 hours in a Dean-Stark apparatus. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was purified by column
chromatography [SiO$_2$: hexanes/EtOAc (9:1, v/v)] to afford 76 (118 mg, 24%) as an orange solid. ESIMS: $m/z = 612.3379$ [M + H]$^+$ ($m/z$ calcd. for C$_{40}$H$_{41}$BF$_2$N$_3 = 612.3363$; $^1$H NMR (CDCl$_3$): $\delta = 1.01$ (3H, t, 8 Hz), 1.86 (3H, t, 8 Hz), 1.32 (3H, s), 1.34 (3H, s), 1.63 (6H, s), 2.34 (4H, q, 8 Hz), 2.61 (3H, s), 7.23–7.40 (5H, m), 7.50 (3H, bs), 7.72 (4H, d, 8 Hz), 7.85 (1H, d, 16 Hz), 8.20 (2H, d, 8 Hz); $^{13}$C NMR (CDCl$_3$): $\delta = 11.7, 12.2, 13.3, 14.6, 14.9, 17.5, 18.7, 25.3, 53.8, 121.3, 122.0, 126.2, 127.6, 128.2, 128.8, 129.1, 129.3, 129.5, 132.4, 132.8, 133.1, 133.6, 133.9, 134.6, 136.1, 138.6, 140.0, 140.1, 148.1, 148.3, 153.6, 157.1, 183.1.

**Synthesis of 79a**

A solution of 84 (417 mg, 1.0 mmol), 16 (300 mg, 1.0 mmol) and trifluoroacetic acid (TFA, 72 µL, 0.967 mmol) in EtOH (10 mL) was heated under reflux for 24 hours. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (15 mL) and washed with H$_2$O (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO$_2$: Hexane/EtOAc (2:1, v/v)] to give 79a (350 mg, 50%) as a red oil. ESIMS: $m/z = 724.4711$ [M + H]$^+$ ($m/z$ calcd. for C$_{45}$H$_{62}$N$_3$O$_5 = 724.4689$); $^1$H NMR (CDCl$_3$): $\delta = 0.91$ (3H, t, 7 Hz), 1.28 (26H, s), 1.59–1.61 (2H, m), 2.28 (2H, t, 7 Hz), 3.02 (3H, s), 3.62 (2H, t, 6 Hz), 4.25 (2H, t, 6 Hz), 7.51 (1H, d, 8 Hz), 4.61 (2H, s), 6.15 (1H, d, 16 Hz), 6.64–6.76 (4H, m), 6.89 (2H, t, 8 Hz), 7.11–7.17 (2H, m), 7.32 (2H, d, 9 Hz), 7.96–8.01 (2H, m); $^{13}$C NMR (CDCl$_3$): $\delta = 13.9, 22.8, 24.2, 25.1, 29.3, 29.6, 29.9, 32.1, 34.1, 38.2, 44.3, 50.7, 61.3, 109.3, 111.2, 111.6, 112.3, 116.4, 117.9, 122.7, 123.1, 123.2, 124.3, 124.6, 125.3, 128.2, 131.7, 133.5, 136.1, 140.7, 141.9, 162.5, 173.2.
Synthesis of 80a

A solution of 87 (71 mg, 0.3 mmol), 44 (120 mg, 0.2 mmol) and TFA (0.5 mL, 6.5 mmol) in EtOH (10 mL) was heated for 4 hours under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (20 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO$_2$: Hexane/EtOAc (3:2, v/v)] to give 80a (24 mg, 14%) as a green solid. ESIMS: $m/z = 721.3959$ [M + H]$^+$ ($m/z$ calcd. for C$_{43}$H$_{53}$N$_4$O$_6$ = 721.3965); $^1$H NMR (CDCl$_3$): $\delta$ = 0.88 (3H, t, 7 Hz), 1.20–1.40 (26H, m), 1.55–1.60 (2H, m), 3.42 (6H, q, 7 Hz), 4.63 (3H, d, 9 Hz), 6.03 (1H, t, 5 Hz), 6.47 (1H, d, 3 Hz), 6.56–6.63 (3H, m), 6.87–6.95 (2H, m), 7.23 (1H, d, 2 Hz), 7.51–7.56 (2H, m), 7.61 (1H, d, 2 Hz), 7.99–8.05 (2H, m).

Synthesis of 81a

A solution of 89 (48 mg, 0.1 mmol), 44 (21 mg, 0.1 mmol) and trifluoroacetic acid (20 mg, 0.2 mmol) in EtOH (20 mL) was heated under reflux for 18 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (20 mL). The resulting solution was washed with H$_2$O (2 × 20 mL), dried over Na$_2$SO$_4$ and then the solvent was distilled off under reduced pressure to yield 81a (55 mg, 80%) as a blue solid. ESIMS: $m/z = 771.3596$ [M + H]$^+$ ($m/z$ calcd. for C$_{42}$H$_{51}$N$_4$O$_{10}$ = 771.3600); $^1$H NMR (CDCl$_3$): $\delta$ = 1.14 (6H, t, 6 Hz), 1.25 (3H, bs), 1.37 (3H, bs), 3.23 (3H, bs), 3.39–3.57 (22H, m), 4.60 (1H, d, 6 Hz), 4.70 (1H, d, 6 Hz), 6.44 (1H, s), 6.62 (1H, d, 9 Hz), 6.64–6.77 (2H, m), 6.90 (1H, d, 9 Hz), 7.10–7.20 (1H, m), 7.28 (1H, d, 9 Hz), 7.60 (1H, d, 6 Hz), 7.76 (1H, s), 7.94 (1H, dd, 3 Hz and 9 Hz), 8.06 (1H, d, 3 Hz).
Synthesis of the hexafluorophosphate salt of 83

A solution of 90 (317 mg, 0.6 mmol) and 44 (146 mg, 0.6 mmol) in EtOH (10 mL) was heated under reflux for 18 h. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (3 mL). The addition of Et$_2$O (20 mL) caused the formation of a precipitate, which was filtered off to give the hexafluorophosphate salt of 83 (40 mg, 94%) as a blue solid. ESIMS: $m/z = 634.3506$ [M – PF$_6$]$^+$ ($m/z$ calcd. for C$_{36}$H$_{48}$N$_3$O$_7$ = 634.3487); $^1$H NMR (CD$_3$CN): $\delta =$ 1.24 (6H, t, 8 Hz), 1.80 (6H, s), 3.23 (3H, s), 3.51–3.65 (20H, m), 3.90 (3H, s), 6.60 (1H, s), 6.85 (1H, d, 8 Hz), 7.51 (1H, d, 8 Hz), 7.65 (1H, d, 8 Hz), 7.85 (1H, d, 16 Hz), 8.02 (1H, d, 8 Hz), 8.13 (1H, s), 8.19 (1H, d, 16 Hz), 8.42 (1H, s); $^{13}$C NMR (CD$_3$CN): $\delta =$ 12.3, 26.2, 34.0, 40.1, 44.7, 45.7, 52.1, 58.3, 69.5, 70.4, 70.5, 70.6, 72.0, 97.1, 110.0, 110.5, 112.0, 113.0, 114.2, 122.1, 129.0, 133.1, 135.3, 143.5, 144.5, 151.2, 151.9, 155.3, 158.8, 160.0, 166.2, 183.0.

Synthesis of 84

A solution of N,N-dicyclohexylcarbodiimide (DCC, 205 mg, 1.7 mmol) in CH$_2$Cl$_2$ (5 mL) was added dropwise over the course of 10 min to a solution of N-methyl-N-(2-hydroxyethyl)-4-aminobenzaldehyde (300 mg, 1.7 mmol), heptadecanoic acid (453 mg, 1.7 mmol) and 4-(dimethylamino)pyridine (DMAP, 205 mg, 1.7 mmol) in CH$_2$Cl$_2$ (20 mL) maintained at 0 ºC under Ar. The reaction mixture was allowed to warm up to ambient temperature and stirred for 24 hours under these conditions. The solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO$_2$, Hexane/EtOAc (1:1, v/v)] to afford 84 (670 mg, 93%) as a yellow solid. ESIMS: $m/z = 432.3471$ [M + H]$^+$ ($m/z$ calcd. for C$_{27}$H$_{46}$NO$_3$ = 432.3478); $^1$H NMR (CDCl$_3$): $\delta =$ 0.89 (3H, t, 6 Hz), 1.27 (26H, s), 1.56–1.59 (2H, m), 2.25 (2H, t, 7 Hz), 3.10 (3H, s), 3.71 (2H, t, 6 Hz), 4.30 (2H, t, 6 Hz), 6.77 (2H, d, 9 Hz), 7.75 (2H, d, 9
Hz), 9.76 (1H, s); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta = 13.9, 22.9, 25.0, 29.3, 29.6, 29.9, 32.2, 34.0, 38.5, 50.7, 61.3, 111.6, 126.1, 131.8, 154.0, 173.1, 189.5\).

**Synthesis of \(86\)**

A solution of DCC (764 mg, 3.7 mmol) in CH\(_2\)Cl\(_2\) (5 mL) was added dropwise over the course of 10 min to a solution of \(85\) (500 mg, 2.5 mmol), \(N\)-hydroxysuccinimide (NHS, 425 mg, 3.7 mmol) and DMAP (30 mg, 0.2 mmol) in CH\(_2\)Cl\(_2\) (80 mL) maintained at 0 °C under Ar. The reaction mixture was allowed to warm up to ambient temperature and was stirred for 15 hours under these conditions. The resulting precipitate was filtered off and \(n\)-decylamine (580 mg, 3.7 mmol) was added dropwise to the filtrate over the course of 10 min. The solution was stirred for 12 hours at ambient temperature. The resulting precipitate was filtered off and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO\(_2\), Hexane/EtOAc (1:2, v/v)] to afford \(86\) (542 mg, 43%) as a white solid. ESIMS: \(m/z = 343.2734\) [M + H]\(^+\) (\(m/z\) calcd. for C\(_{22}\)H\(_{35}\)N\(_2\)O = 343.2749); \(^1\)H NMR (CDCl\(_3\)): \(\delta = 0.89\) (3H, t, 6 Hz), 1.28–1.36 (20H, m), 1.62–1.73 (2H, m), 2.32 (3H, s), 3.47 (2H, q, 6 Hz), 6.18 (1H, bs), 7.54 (1H, d, 8 Hz), 7.66 (1H, dd, 2 and 8 Hz), 7.81 (1H, d, 2 Hz); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta = 14.0, 15.5, 22.6, 22.8, 27.0, 29.2, 29.3, 29.5, 29.7, 31.8, 40.2, 53.8, 119.4, 120.8, 126.4, 131.8, 146.0, 156.2, 157.2, 167.7.

**Synthesis of \(87\)**

A solution of \(86\) (342 mg, 1.2 mmol) and 4-nitro-2-chloromethyl-phenol (225 mg, 1.2 mmol) in MeCN (20 mL) was heated for 24 hours under reflux and Ar. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH\(_2\)Cl\(_2\) (15 mL) and washed with H\(_2\)O (20 mL). The organic phase was dried over Na\(_2\)SO\(_4\) and the solvent was distilled off under reduced pressure and the residue was purified by column chromatography [SiO\(_2\), Hexane:EtOAc (2:1, v/v)] to
afford 87 (180 mg, 36%) as a white solid. ESIMS: \( m/z = 494.2987 \) [M + H]⁺ (\( m/z \) calcd. for C\(_{29}\)H\(_{40}\)N\(_3\)O\(_4\) = 494.3019); \(^1\)H NMR (CDCl\(_3\)): \( \delta = 0.87 \) (3H, t, 7 Hz), 1.18–1.30 (20H, m), 1.54–1.66 (5H, m), 3.39 (2H, q, 6 Hz), 4.65 (2H, s), 6.21 (1H, t, 6 Hz), 6.55 (1H, d, 8 Hz), 6.72 (1H, d, 9 Hz), 7.50 (1H, dd, 2 and 8 Hz), 7.62 (1H, d, 2 Hz), 7.95 (1H, dd, 3 and 9 Hz), 8.07 (1H, d, 3 Hz); \(^{13}\)C NMR (CDCl\(_3\)): \( \delta = 13.9, 16.2, 18.5, 22.8, 27.3, 29.6, 29.8, 30.1, 32.1, 34.0, 39.9, 48.1, 103.7, 108.3, 118.2, 121.9, 124.6, 124.7, 127.9, 128.5, 138.6, 141.1, 150.1, 159.2, 167.0.

**Synthesis of 88**

DCC (405 mg, 2.0 mmol) was slowly added to a solution of 85 (200 mg, 1.0 mmol), NHS (227 mg, 2.0 mmol) and DMAP (25 mg, 0.2 mmol) in CH\(_2\)Cl\(_2\) (30 mL) maintained at 0 °C under Ar. The mixture was allowed to warm up to ambient temperature, stirred under these conditions for 15 h and filtered. 2-(2-(2-(methoxyethoxy)-ethoxy)-ethoxy)-ethylamine (400 mg, 2.0 mmol) was added to the filtrate and the resulting solution was stirred for 12 h at ambient temperature. The solvent was distilled off under reduced pressure to give 88 (300 mg, 90%) as a yellow oil. ESIMS: \( m/z = 393 \) [M + H]; \(^1\)H NMR (CDCl\(_3\)): \( \delta = 1.19 \) (6H, s), 2.56 (3H, s), 3.18 (2H, t, 4 Hz), 3.23 (2H, t, 4 Hz), 3.40–3.54 (15 H, m), 7.43 (1H, d, 8 Hz), 7.65 (1H, t, 6Hz), 7.75 (1H, d, 8 Hz).

**Synthesis of 89**

A solution of 88 (100 mg, 0.3 mmol) and 2-chloromethyl-4-nitrophenol (48 mg, 0.3 mmol) in MeCN (10 mL) was heated for 24 hours under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH\(_2\)Cl\(_2\) (20 mL). The resulting solution was washed with H\(_2\)O (20 mL), dried over Na\(_2\)SO\(_4\) and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography (SiO\(_2\): CHCl\(_3\)/MeOH (99:1 → 95:5, v/v) to afford 89 (80 mg, 59%) as a yellow oil. ESIMS: \( m/z = 544 \) [M + H]; \(^1\)H NMR
(CD$_3$CN): $\delta = 1.54$ (3H, s), 1.59 (3H, s), 2.40 (3H, s), 3.28 (3H, s), 3.34–3.52 (16H, m), 4.59 (1H, d, 16 Hz), 4.73 (1H, d, 6 Hz), 6.69 (1H, d, 8 Hz), 6.73 (1H, d, 8 Hz), 7.12 (1H, bs), 7.58 (1H, d, 8 Hz), 7.63 (1H, s), 7.93 (1H, d, 8 Hz), 8.14 (1H, s); $^{13}$C NMR (CD$_3$CN): $\delta = 16.8, 18.9, 26.1, 40.4, 40.5, 48.7, 58.8, 70.2, 70.9, 71.1, 72.5, 104.1, 108.9, 118.6, 120.5, 22.5, 124.6, 124.9, 127.8, 128.5, 139.4, 141.5, 150.8, 159.7, 167.8.

**Synthesis of the hexafluorophosphate salt of 90**

A solution of 88 (314 mg, 0.8 mmol) and methyl iodide (0.5 mL, 8 mmol) in MeCN (20 mL) was heated for 24 hours under reflux. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ (3 mL). The addition of Et$_2$O (20 mL) caused the formation of a precipitate, which was filtered off and dissolved in Me$_2$CO (5 mL). After the addition of a saturated aqueous solution of NH$_4$PF$_6$ (5 mL), the mixture was concentrated under reduced pressure to half of its original volume and the resulting suspension was extracted with CH$_2$Cl$_2$. The organic phase was dried over Na$_2$SO$_4$ and the solvent was distilled off under reduced pressure to give the hexafluorophosphate salt of 90 (317 mg, 74%) as dark red oil. ESI MS: $m/z = 407$ [M – PF$_6$]$^+$; $^1$H NMR (CDCl$_3$): $\delta = 1.56$ (6H, s), 2.75 (3H, s), 3.27 (3H, s), 3.45–3.47 (2H, m), 3.52–3.73 (14H, m), 3.98 (3H, s), 7.80 (1H, d, 8 Hz), 8.05 (1H, d, 8 Hz), 8.13 (1H, s); $^{13}$C NMR (CDCl$_3$): $\delta = 14.9, 22.3, 35, 40.8, 55.7, 5.89, 70.0, 70.2, 70.9, 71.1, 72.5, 72.6, 116.1, 123.2, 129.6, 137.1, 142.9, 145.1, 167.0, 167.1, 199.5.

**Polymer Micelles**

A solution of 51 (2.5 mg mL$^{-1}$, 200 µL) in CHCl$_3$ was added to a solution of 77, 78, 79, 39 or 80 (0.1 mg mL$^{-1}$, 50 µL) in CHCl$_3$. Alternatively, a solution of 51 (2.5 mg mL$^{-1}$, 200 µL) in CHCl$_3$ was mixed with solutions of 77 (0.1 mg mL$^{-1}$, 20 µL) and 78, 79, 39 or 80 (0.1 mg mL$^{-1}$, 100 µL) in CHCl$_3$. Each mixture was heated at 40 °C in an open vial.
After the evaporation of the solvent, the residue was purged with air and dispersed in PBS (1 mL, pH = 7.0). After vigorous shaking, the dispersion was filtered and the filtrate was used for the spectroscopic and imaging experiments without further purification.
REFERENCES


171 The absorbance at 390 nm (b in Figure 2.13) together with the molar extinction coefficient of the model phenolate (d in Figure 2.13) indicate that the addition of 100 eq. of tetrabutylammonium hydroxide converts ca. 3% of 15a into the corresponding hemiaminal (d in Figure 2.7).


184 Compound 36a was originally designed to enhance coloration efficiency and its photochromism was previously reported (ref. 170f,g). Compound 39a was described in a preliminary communication (ref. 170n).

185 Compounds 36a and 38a are not sufficiently soluble in deuterated acetonitrile to perform variable-temperature 1H NMR spectroscopic studies.

186 The rate constant (k1) for the degenerate site exchange at a given temperature (T) was determined with equation (1) from the line width (Δν1/2) at half maximum of the resonance for MeA and MeB, measured at T, and the frequency separation (ΔνL) at the stopped-exchange limit (Nelson, J. H. *Nuclear Magnetic Resonance Spectroscopy*; Prentice-Hall: Upper Saddle River, 2003). ΔνL is the frequency separation between the two singlets expected for the two sets of diastereotopic methyl protons when the degenerate site-exchange process is not occurring on the
1H NMR timescale. In principle, \( \Delta \nu_L \) should be measured at a sufficiently low \( T \) to prevent exchange. However, both \( 37a \) and \( 39a \) are not sufficiently soluble in deuterated acetonitrile at temperature lower than 0 °C to permit the direct determination of this parameter. As a result, \( \Delta \nu_L \) was estimated from the 1H NMR spectrum of the model compound \( 16 \) recorded at –35 °C.

\[
k_1 = \frac{\pi \Delta \nu^2}{2 \Delta \nu_{1/2}}
\]  

The enthalpy of activation (\( \Delta H^\ddagger \)) and the entropy of activation (\( \Delta S^\ddagger \)) were determined from the slope and intercept of the plot of ln \( \left( \frac{k_1}{T} \right) \) against \( T^{-1} \), according to equation (2). The free-energy of activation (\( \Delta G^\ddagger \)) was determined with equation (3).

\[
\ln \frac{k_1}{T} = 23.76 - \frac{\Delta H^\ddagger}{R T} + \frac{\Delta S^\ddagger}{R}
\]

\[
\Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger
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188 The ratios between the ring-closed species \( 36a-38a \) and their ring-open isomers \( 36b-38b \) in methanol at 20 °C are 90:10, 98:2 and 97:3 respectively at equilibrium.

189 The excitation of \( 39a \), embedded in a PMMA matrix, also results in the formation of \( 39b \) with the concomitant appearance of a transient absorption in the visible region. Under these conditions, however, the reisomerization kinetics are significantly slower than in acetonitrile and the lifetime of \( 39b \) is 77 ms.


The relative concentrations of the two isomers of each system at equilibrium were estimated from the absorbance of the 3H-indolium chromophore of 78b and 79b in the visible region, using the molar extinction coefficient of the model compound 82.

The pKa of 4-nitrophenol is 7.15 Haynes, W. M. (Ed.) CRC Handbook of Chemistry and Physics; CRC Press/Taylor and Francis: Boca Raton, 2012. This value suggests that a significant fraction of the ring-open isomer has the 4-nitrophenolate anion in a protonated form.

The relative concentrations of the two isomers at equilibrium were estimated from the absorbance of the 3H-indolium chromophore of 81b in the visible region, using the molar extinction coefficient of the model compound 83.

The fluorescence quantum yield of the hexafluorophosphate salt of 49 is 0.09 in acetonitrile170o and 0.04 in PBS (pH = 7.0). Thus, the transition from organic to aqueous solutions tends to discourage the radiative deactivation of this particular fluorophore. In the presence of the amphiphilic co-polymer 51, however, the quantum yield in PBS increases to 0.13. These observations suggest that the polymeric host limits the exposure of the fluorescent guest to the aqueous environment.


The synthesis of 16 was originally reported in ref. 195.
