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Fluorescent Dyes

Molecular Insight into Long-Wavelength Fluorogenic Dye Design: Hydrogen Bond Induces Activation of a Dormant Acceptor

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Abstract: The detection of chemical or biological analytes in response to molecular changes relies increasingly on fluorescence methods. Therefore, there is a substantial need for the development of improved fluorogenic dyes. In this study, we demonstrated how an intramolecular hydrogen bond activates a dormant acceptor through a charge induction between phenolic hydrogen and a heteroaryl nitrogen moiety. As a result, a new fluorochrome is produced, and the molecule exhibits a strong fluorescent emission. When the strength of the hydrogen bonding was increased by conformational locking, the obtained dye emitted at longer wavelengths and fluoresced under physiological conditions. The dye was implemented in a turn-ON system responsive to hydrogen peroxide. The molecular insight provided by this study should assist in the design of fluorescent dyes that are suitable for in vitro and in vivo applications.

The detection of chemical or biological analytes, in response to molecular changes,[1,2] relies increasingly on fluorescence.[3–6] Thus, there is a demand for more sensitive, more specific, and more versatile fluorescent molecules that can be used for in vitro and in vivo applications.[7–9] Such molecules should have a high physiological stability, high quantum yields of fluorescence, long emission wavelengths, large Stokes shifts, and a good photostability.[10–12] We have recently developed a novel class of turn-ON cyanine-based probes with a long-wavelength fluorescence emission.[13] The probes are based on the fluorochrome QCy7, which is generated upon removal of a specific trigger moiety by an analyte of interest. Upon triggering, a unique change in the π-electron system leads to generation of a cyanine dye with strong near-infrared (NIR) fluorescence. In a representative example, the probe enabled detection of endogenous hydrogen peroxide, produced in an acute inflammation model in mice. The modular structure of the QCy7 fluorochrome enabled us to develop a library of dye compounds that can be used for turn-ON probe design.[14,15]

It is tremendously difficult to predict the fluorescence characteristics of a given dye, since there are numerous pathways for non-radiative decay in which an excited molecule can lose its energy. Non-radiative electron decay can be significantly decreased by locking the excited molecule in a planar conformation. Here we report a new study that provides molecular insight into an intramolecular hydrogen-bond bridge, between a donor and a dormant acceptor. By conjugation with an additional acceptor, the hydrogen bonding is harnessed to produce a long-wavelength fluorogenic dye.

The general molecular structure of a dye activated through hydrogen bonding is illustrated in Figure 1. The dye is composed of a latent phenol donor conjugated at the *para* position to an active acceptor moiety and at the *ortho* position to a dormant acceptor (structure **I**). Upon formation of an intramolecular hydrogen bond between the donor and the dormant acceptor (**II**), three functions are achieved: 1) the dormant acceptor gains a positive charge enabling it to act as an active acceptor, 2) the phenol latent donor gains a negative charge allowing it to act as an active donor, and 3) the hydrogen bridge locks the molecule in a planar conformation. At the same time, an intramolecular charge transfer (ICT) from the phenol donor to either one of the two acceptors forms a new donor–acceptor pair with longer π-electron conjugation (**III** and **IV**). As a result, a long-wavelength-emitting fluorochrome is formed.

To test the proposed hydrogen-bridge formation mode of action of a dormant acceptor, we prepared dyes **1** and **2** (Figure 2). Dye **1** is composed of the indolium general acceptor (blue) and 2-pyridine moiety (green); the 2-pyridine is capable of forming a hydrogen bond with the phenol donor (structure **1′**). Dye **2** is an analogue of dye **1** in which the 2-pyridine moiety is replaced with a 4-pyridine. The hydrogen bond cannot form in dye **2** and, therefore, dye **2** served as a negative control for the hydrogen-bridge activation pathway expected for dye **1**.

The synthesis of dyes **1** and **2** was achieved as outlined in Figure 3. Compound **1a** was subjected to Miyaura borylation using bis(pinacolato)diboron, tricyclohexylphosphine as a ligand, and bis(dibenzylideneacetone)palladium(0) as a catalyst to obtain the aryl boronic ester **1b**. Ester **1b** was then coupled with 2-bromopyridine using tetrakis(triphenylphosphine)-palladium(0) as a catalyst by the Suzuki cross-coupling reaction to afford compound **1c**. In order to prepare compound **2a**,

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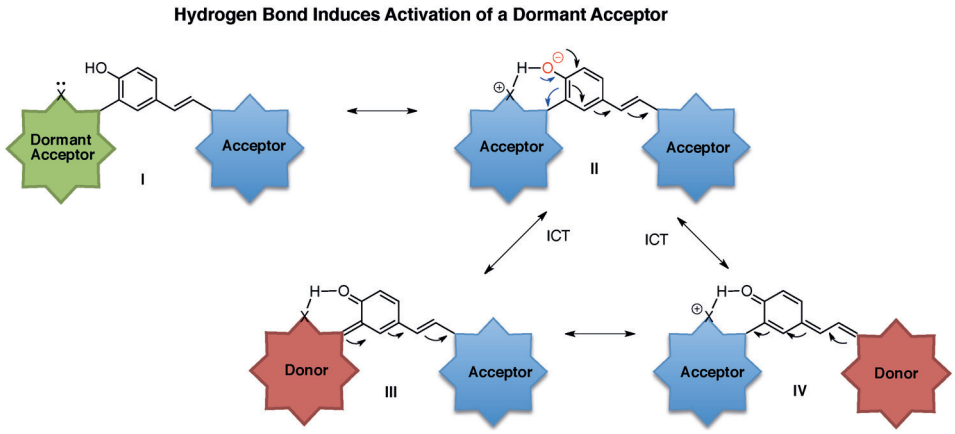

Figure 1. Graphical illustration of a dormant acceptor activated by hydrogen bonding to produce a new long-wavelength emitting fluorochrome.

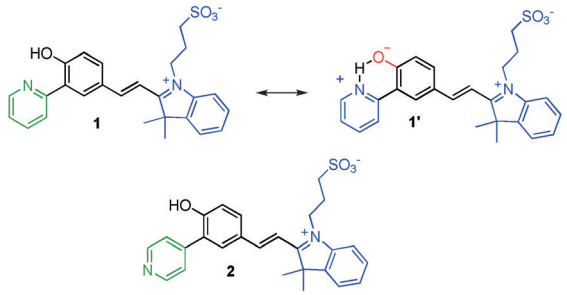

Figure 2. 2-Pyridine dormant acceptor (structure **1**) and negative control 4-pyridine (structure **2**) used to validate the hydrogen-bridge activation.

the boronic ester **1b** was coupled with 4-bromopyridine hydrochloride using similar Suzuki cross-coupling conditions. Finally, the Suzuki cross-coupling products **1c** and **2a** were condensed with indolium propanesulfonate acceptor **1d** to afford dyes **1** and **2**, respectively.

The absorbance spectra of the dyes **1** and **2** measured in acetonitrile were almost identical with maxima at 450 nm (Figure 4). However, the fluorescence spectra of the two dyes were entirely different. Dye **2** exhibited a minor fluorescence emission with a λ_{max} of 520 nm, whereas dye **1** showed a strong fluorescence emission with a λ_{max} of 610 nm ($\Phi =$ 10.5%). This result provides support for our suggested dormant-acceptor activation concept in which a hydrogen bridge formed in dye **1** turns on the pyridine dormant acceptor and thereby produces an active fluorochrome. Such a structure cannot be formed in control dye **2** and, therefore, no substantial fluorescence was observed.

To assess whether the hydrogen bridge of dye **1** is formed under physiological conditions, we measured the pK_a of the phenols in dyes **1** and **2**. The pK_a values were determined by monitoring absorbance as a function of pH (Figure 5). The phenol of dye **1** had a pK_a of 8.0, significantly higher than that of dye **2** (pK_a of 6.4). This difference, which could be observed through changes of color, is attributed to the hydrogen bond formation with the nitrogen of the 2-pyridine ring of dye **1**; such a hydrogen bond cannot be formed in dye **2**.

The pK_a value of 8.0 measured for dye **1** is not sufficient to maintain a hydrogen bond under physiological conditions at pH 7.4. Furthermore, dye **1** exhibited fluorescence emission with a λ_{max} of 610 nm. This wavelength is below the optimal NIR window suitable for in vivo use. To further increase the strength of the intramolecular hydrogen bond and the increase the emission wavelength, we sought to design a molecular structure with a "carbon bridge" between the phenol moiety and the pyridine dormant acceptor (Figure 6). We reasoned that this modification would lock the molecule in a planar conformation and thereby increase the strength of the hydrogen bond. In addition, the planarization of the molecule should minimize non-radiative electron decay of the excited state, thus increasing the fluorescence quantum yield and yielding fluorescence at longer wavelengths.

Based on the design presented in Figure 6, we chose to prepare dye **3** (Figure 7). The molecular structure of the dye includes a conjugated carbon bridge between the two aryl rings and a conjugated indolium acceptor at the *ortho* position of the hydroxylphenol. The nitrogen atom of the quinoline moiety is capable of forming a hydrogen bond with the phenolic hydrogen as illustrated in Figure 7. As described for the pyridine-based dye, an ICT from the phenol donor to either one of the two acceptors will form a new donor–acceptor pair with elongated π-electron conjugation.

The synthesis of dye **3** was achieved as shown in Figure 8. Commercially available 10-hydroxybenzoquinoline was *ortho*-formylated using paraformaldehyde and magnesium chloride to afford aldehyde **3a**, which was condensed with indolium acceptor **1d** to afford dye **3**.

The absorbance and the fluorescence spectra of dye **3** were initially measured in acetonitrile (Figure 9). Benzoquinoline dye **3** exhibited a notable redshift in comparison to dye **1**. The maximum absorbance wavelength of dye **3** was 480 nm and the emission wavelength was 670 nm. In addition, the observed fluorescence intensity was also notably higher than that of pyridine-based dye **1** ($\Phi =$15.7%). These changes are attributed to the extended conjugation and the stronger hydrogen bond in dye **3** relative to dye **1**. The fluorescence emission of dye **3** is well within the NIR window, making it a candidate for in vivo imaging applications. However, under aqueous conditions, the dormant-acceptor activation mode of action proposed in Figure 7 will be ON only if the intramolecular hydrogen bond is preserved at physiological pH.

As predicted, the measured pK_a of dye **3** was significantly higher than that of dye **1**, with a value of 11.3 (see the Supporting Information for details). A phenol with this pK_a value should be able to preserve the hydrogen bond also under

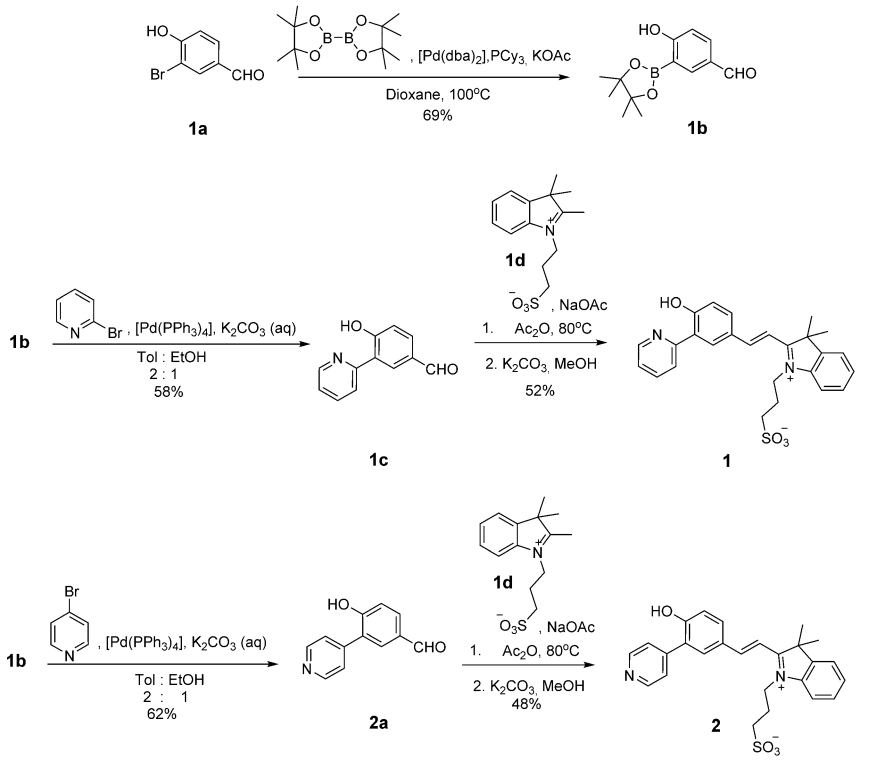

Figure 3. Chemical synthesis of dyes **1** and **2**.

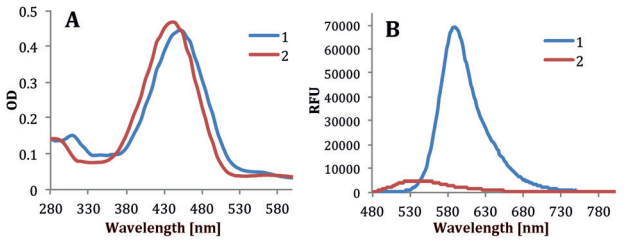

Figure 4. A) Absorbance and B) fluorescence spectra of dyes **1** and **2** [50 μM] in acetonitrile.

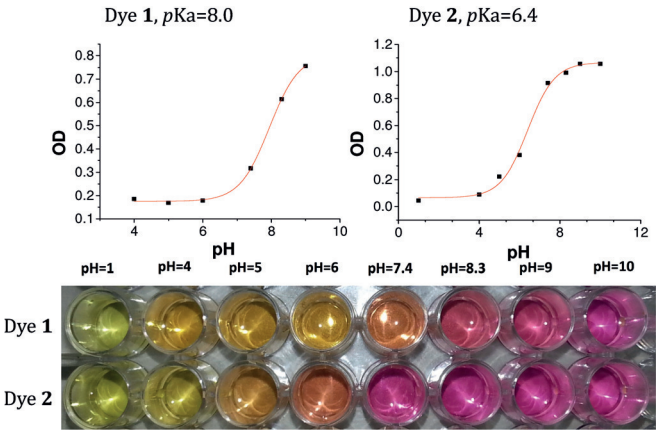

Figure 5. Measurement of pK_a values for dyes **1** and **2** (λ_{max} 540 nm).

physiological conditions. Indeed, the observed fluorescent emission of dye **3** at pH 7.4 indicates that the hydrogen bridge is formed (Figure 10). Dye **3** also showed a substantial NIR fluorescence emission in aqueous buffer at physiological pH.

As illustrated in Figure 7, the dormant-acceptor activation mechanism of dye **3** relies on the hydrogen bridge formation between the phenol and the quinolone nitrogen atom. Therefore, masking of the phenol by a protecting group should eliminate the hydrogen bridge and the corresponding fluorochrome. This approach is commonly used to construct turn-ON fluorescent probes, where the masking is performed with an analyte-responding group. To demonstrate such a probe based on benzoquinoline dye **3**, we evaluated the turn-ON behavior of a probe obtained through masking of the phenol moiety by a benzyl boronate ester protecting group (Figure 11; **4**). This group can be removed by a reaction with hydrogen peroxide through an oxidation–elimination sequence. Incubation of hydrogen peroxide with probe **4** should result in the formation of intermediate **4a**. The latter should then undergo 1,6-quinone-methide elimination to release the active form of dye **3**.

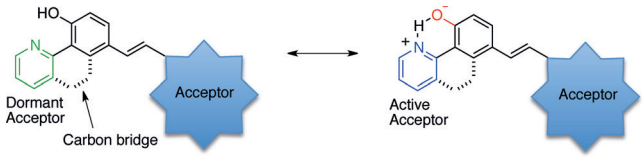

Figure 6. Molecular structure of dye with pyridine as a dormant acceptor fused through a carbon bridge.

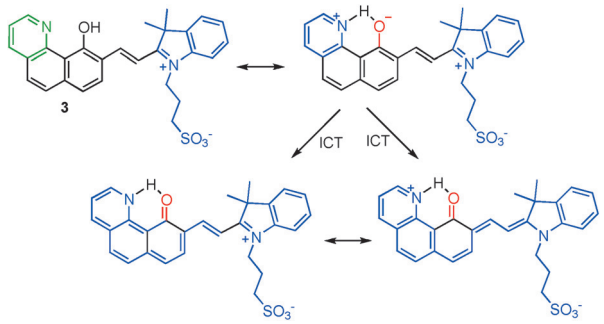

Figure 7. Activation of bridged quinoline dormant acceptor by hydrogen bonding to produce a new long-wavelength emitting fluorochrome.

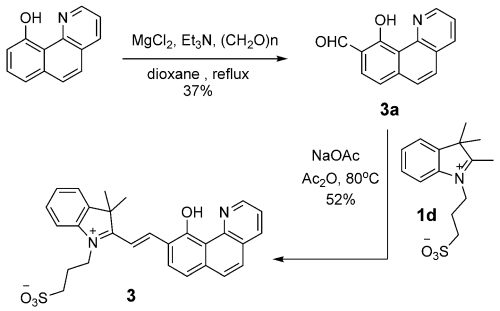

Figure 8. Chemical synthesis of dye **3**.

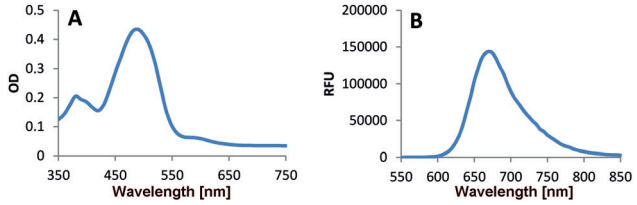

Figure 9. A) Absorbance (λ_{ex} 480 nm) and B) fluorescence (λ_{em} 670 nm) spectra of dye **3** [50 μM] in acetonitrile.

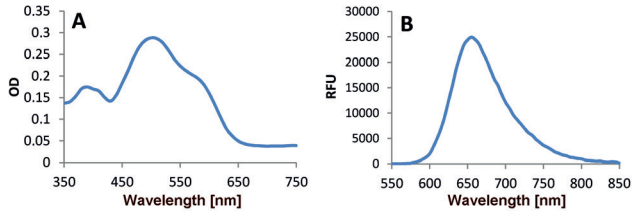

Figure 10. A) Absorbance (λ_{ex} 500 nm) and B) fluorescence spectra (λ_{em} 650 nm) of dye **3** [50 μM] in PBS at pH 7.4.

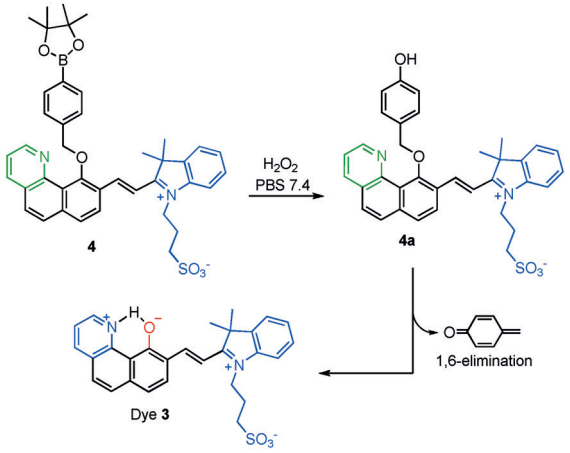

Figure 11. Mechanism of hydrogen peroxide activated NIR fluorescent turn-ON based on dye **3**.

The synthesis of probe **4** was achieved as illustrated in Figure 12. The phenol moiety of compound **3a** was alkylated with benzyl iodide **4b** to produce compound ether **4c**, which was reacted with indolium **1d** to afford probe **4**.

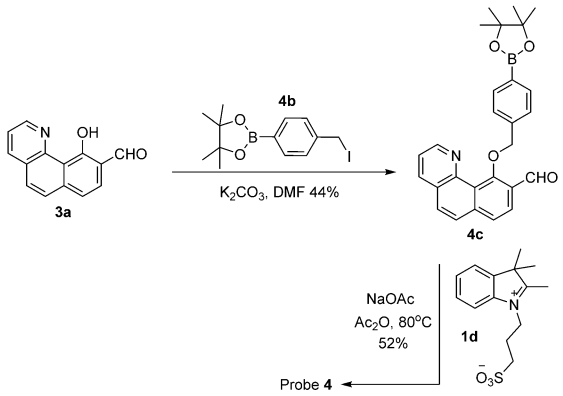

Figure 12. Chemical synthesis of probe **4**.

The fluorescence emission spectra of probe **4** versus that of dye **3** in phosphate-buffered saline (PBS) at pH 7.4 are presented in Figure 13A. Dye **3** shows strong emission with a maximum wavelength of 650 nm, whereas probe **4** exhibits a minor emission in this wavelength range. Incubation of hydrogen peroxide with probe **4** resulted in a significant increase of fluorescent signal, whereas no increase was observed upon incubation in the absence of the hydrogen peroxide (Figure 13B). These results demonstrate implementation of a turn-ON, long-wavelength fluorochrome probe based on the hydrogen-bridge activation pathway.

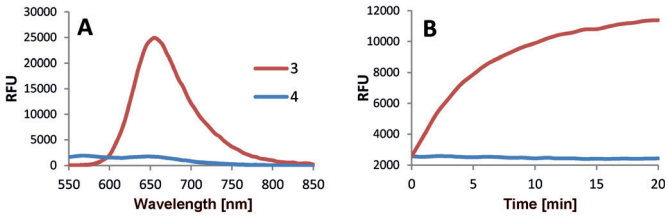

Figure 13. A) Fluorescence spectra (λ_{ex} 480 nm) of probe **4** (blue) and dye **3** (red; [50 μM]) in PBS at pH 7.4; B) fluorescence emission (λ_{em} 650 nm) of probe **4** [50 μM] in the presence (red) and in the absence (blue) of hydrogen peroxide [100 μM].

Intramolecular hydrogen bonding contributes to the photochemical process known as the excited-state intramolecular proton transfer (ESIPT).[16–18] Although, this phenomenon has been characterized in studies of fluorogenic dyes[19–21] and photoacids,[22] there is a lack of information on the influence of hydrogen bond strength and pK_a on the fluorescent properties (wavelength emission and fluorescence intensity) of fluorogenic dyes. Interestingly, the hydrogen-bonding interaction in the excited state of a green fluorescence protein (GFP)-like chromophore led to fluorescence quenching due to a rapid internal conversion or proton/electron transfer.[23] In another related example, an intramolecular hydrogen bond produced a xanthene dye with NIR fluorescence emission.[24]

In this study, we demonstrated that an intramolecular hydrogen bond can activate a dormant acceptor through a charge induction between phenolic hydrogen and a heteroaryl nitro-

gen moiety. The activated acceptor can then form a new elongated π-electron pattern in conjugation with a donor moiety from a separate location on the molecule. The intramolecular hydrogen bond also induces conformational locking of the conjugated backbone. Such an effect is known to reduce non-radiative decay of excited electrons. We increased the hydrogen bond strength through bridging of the molecular skeleton in the benzoquinoline. This improved the conjugation and planarization of the dye molecule and, consequently, shifted the fluorescence to a longer emission wavelength in the NIR region. Due to the increased strength of the hydrogen bond (pK_a of 11.3) in the benzoquinoline molecule, the resulting dye strongly fluoresced under physiological conditions (see the Supporting Information for fluorescence quantum yields). The dye was effectively implemented in a turn-ON probe by masking of the phenol moiety with a responsive substrate for hydrogen peroxide. We anticipate that the molecular insights provided by this study will assist in the design of fluorescent dyes that can be used for in vitro and in vivo applications.

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