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New repertoire of 'donor-two-acceptor' NIR fluorogenic dyes

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ABSTRACT

Dye molecules with various fluorescent wavelengths are widely used for diagnostic and optical imaging applications. Accordingly, there is a constant demand for fluorogenic dyes with new properties. We have recently developed a novel strategy for the design of long-wavelength fluorescent dyes with a turn-ON option. The design is based on a donor-two-acceptor π-electron system that can undergo an internal charge transfer to form a new fluorochrome with an extended π-conjugated system. Here, we describe a series of such dyes based on two novel latent donors, naphthol and hydroxycoumarin. One of the dyes has showed excellent near-infrared fluorescent characteristics and specifically was demonstrated as a mitochondrial imaging reagent in live cells. This unique strategy for fluorogenic dye design has opened new doors for further near-infrared fluorescence probe discovery.

1. Introduction

Fluorescent probes are of considerable interest due to their simple implementation, high sensitivity and high spatial resolution.[1–5] Such probes, based on dye molecules that fluoresce at various wavelengths, are widely used for diagnostic and optical imaging applications.[6–13] We have recently developed a novel strategy for the design of long-wavelength fluorogenic dyes with a turn-ON option.[14] Our design is based on a donor-two-acceptor π-electron system that can undergo an internal charge transfer to form a new fluorochrome with an extended π-conjugated system. This strategy was demonstrated by synthesis of a library of dyes that had fluorescence emission in the near-infrared (NIR) region.[15,16,14b] This optical range is particularly significant for in vivo imaging applications, since live tissues have minimal absorbance and emission in such wavelengths and the NIR photons have better penetration abilities.[17]

Our donor-two-acceptor dye system is composed of a phenol moiety that functions as a latent donor conjugated with two acceptors (Fig. 1). Deprotonation of the phenol leads to formation of a phenolate active donor **III** that is able to donate a pair of π-electrons to either one of the conjugated acceptors (structures **I** and **II**). This intramolecular charge transfer (ICT) generates a resonance species with a π-electron pattern similar to that of Cy7 fluorochrome. The donor capability of the phenolate species **III** can be masked either by a proton or by a specific protecting group. Such a protected phenol can be used as a molecular probe for detection or imaging of an analyte that reacts with the probe to remove the protecting group. The π-electron arrangement of phenolate **III** can be viewed as a general donor-two-acceptor system that can be modified to prepare probes with a turn-ON option. This concept was exemplified by preparing a novel NIR turn-ON probe for detection of hydrogen peroxide. The probe was successfully used to image induced inflammation in mice.[14a]

All of the dyes in our library were based on a phenol donor moiety with various substituents and acceptor moieties. It is also possible to use donors with extended conjugation of π-electron system. Here we report a new series of donor-two-acceptor fluorogenic dyes based on naphthol and on hydroxycoumarin donor nuclei; some have excellent optical qualities.

2. Results and discussion

Three dyes based on a naphthol donor were designed each with two indolium acceptors (Fig. 2). The dyes were synthesized, using our previously published procedure[14], by condensation of 2 equiv of the indolium with the corresponded naphthol-dialdehyde.

Dye **1** was synthesized, as outlined in Figure 3, starting from commercially available 1-naphthol. 1-Naphthol was treated with hexamethylenetetramine (HMT) in trifluoroacetic acid (TFA) via the Duff reaction to afford dialdehyde **1a**. The latter was condensed with 2 equiv of indolium **1b** to afford dye **1**.

Dye **2** was similarly synthesized starting from compound **2a**, which was treated with hexamethylenetetramine to give dialdehyde **2b** (Fig. 4). The latter was condensed with 2 equiv of indolium **1b** to afford dye **2**.

Dye **3** was synthesized as shown in Figure 5. Commercially available 5,7-dimethyl-tetralone was treated with lithium diisopropylamine followed by addition of trimethyl silyl chloride

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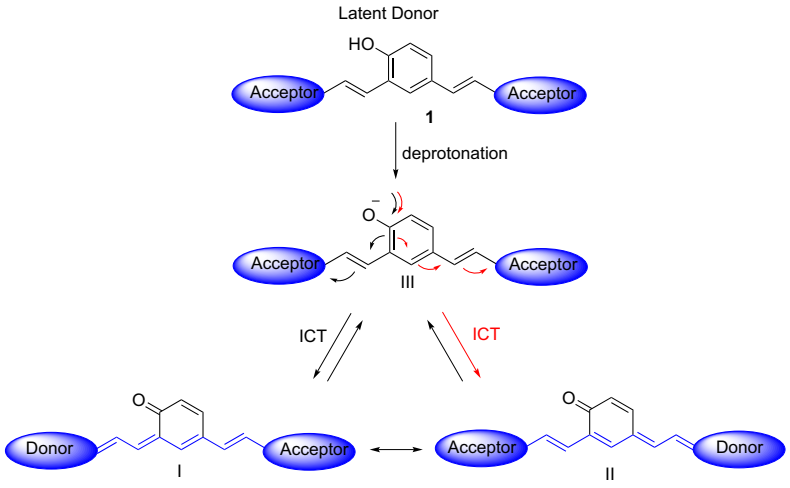


Figure 1. General mechanism of activation of donor-two-acceptor cyanine-like fluorescence probes.

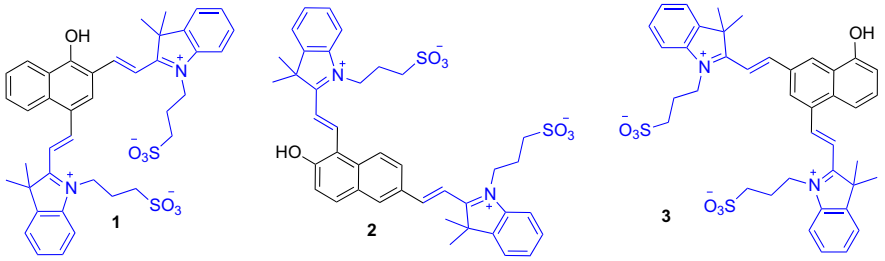


Figure 2. Chemical structures of donor-two-acceptor dyes composed of naphthol donor and two indolium acceptors.

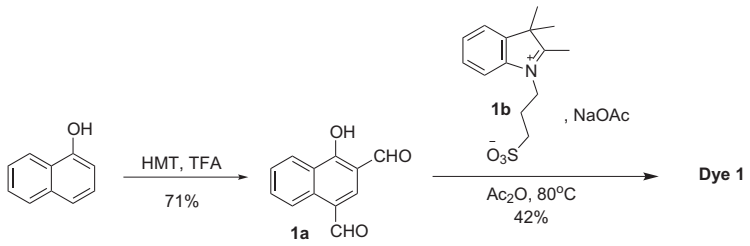


Figure 3. Chemical synthesis of dye **1**.

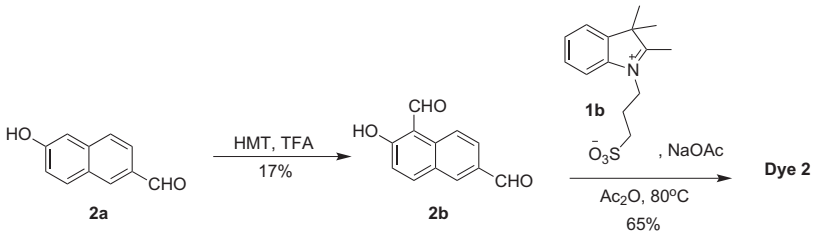


Figure 4. Chemical synthesis of dye **2**.

to afford enol **3a**. The latter was oxidized with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, followed by deprotection of the trimethylsilyl group with tetrabutylammonium fluoride to give naphthol **3b**. The naphthol was reacted with pivaloyl chloride to afford ester **3c**, which was then reacted with *N*-bromosuccinimide to give dibromide **3d**. The dibromide was hydrolyzed in the presence of silver sulfate to afford diol **3e**, which was then oxidized with manganese oxide to give dialdehyde **3f**. The latter was deprotected with potassium carbonate and then condensed with 2 equiv of indolium **1b** to afford dye **3**.

The naphthol donor is composed of two fused aromatic rings; one of the rings has a hydroxyl functional group. Dye **1** has two indolium acceptors on the phenolic moiety, dye **2** has one indolium on the phenol ring and the other on the second ring, and dye **3** has the two indolium acceptors on the non-phenolic ring. The indolium moieties are located at conjugated positions

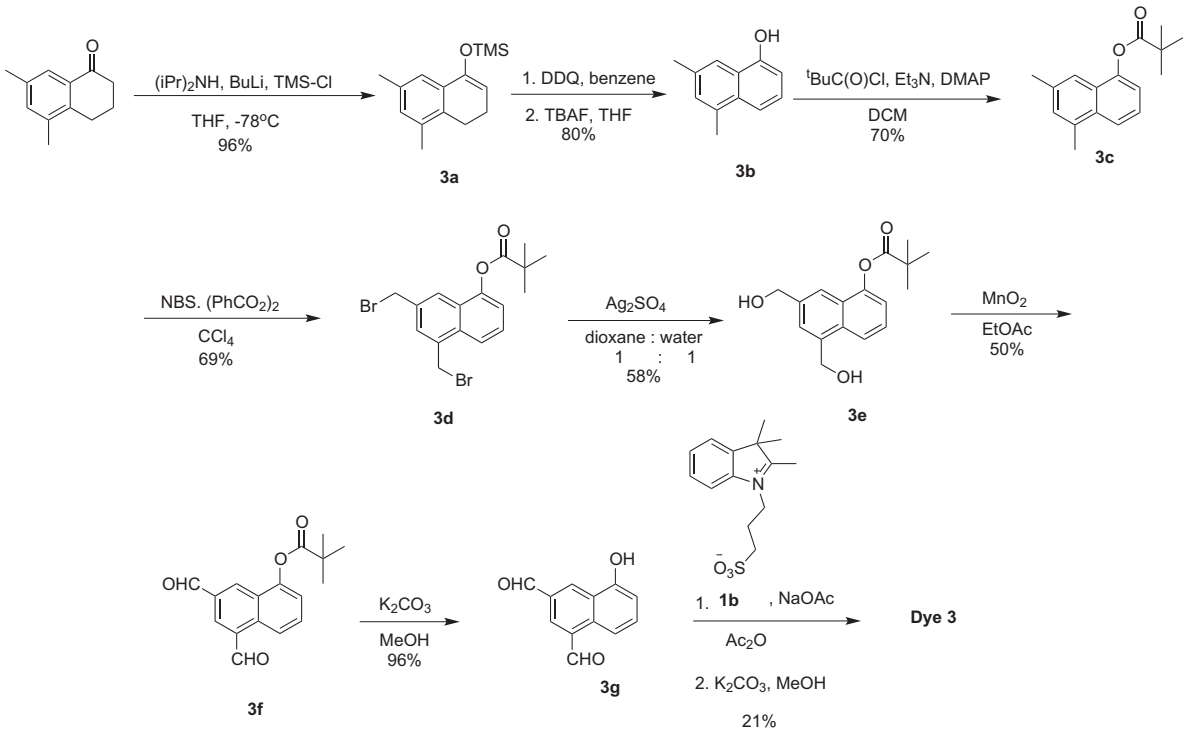


Figure 5. Chemical synthesis of dye **3**.

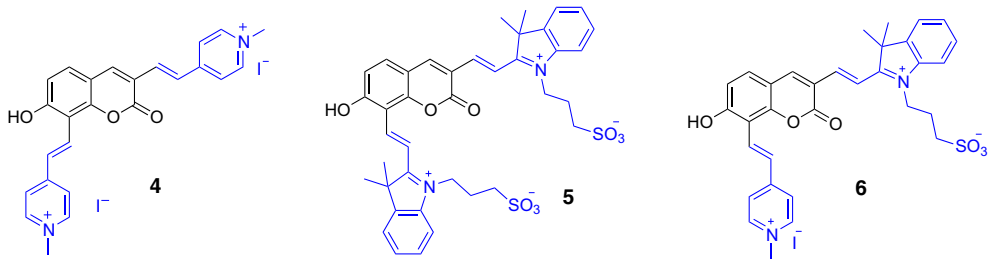


Figure 6. Chemical structures of donor-two-acceptor dyes composed of hydroxycoumarin donor and indolium or picolinium acceptors.

relative to the phenol donor. Thus, upon deprotonation, the obtained phenolate can donate an electron-pair through an intramolecular charge transfer to either one of the indolium acceptors and thereby to form donor-two-acceptor dye system, as illustrated in Figure 1.

An additional three dyes were prepared based on hydroxycoumarin donor with indolium or picolinium acceptors (Fig. 6). These dyes were synthesized by condensation of the corresponded dialdehyde with the indolium or picolinium moieties to form donor-two-acceptor dye systems. Dyes **4** and **5** are composed of either two picolinium or two indolium acceptors, each on different ring of the donor. Dye **6** has one picolinium acceptor on the phenolic ring and one indolium on the lactone ring.

The synthesis of dyes **4** and **5** was achieved as outlined in Figure 7. Coumarin derivative **4a** was treated with hexamethylenetetramine via the Duff reaction to afford aldehyde **4b**. The latter was reacted with osmium tetroxide in the presence of *N*-oxide-*N*-methyl-morpholine to give diol **4c** and then oxidized with sodium periodate to afford dialdehyde **4d**. The dialdehyde was condensed with 2 equiv of *N*-methyl-4-picolinium **4e** to afford dye **4**. Similarly, dye **5** was prepared by condensation of dialdehyde **4d** with 2 equiv of indolium **1b**.

Dye **6** was synthesized as outlined in Figure 8. Dialdehyde **4d** was condensed with 1 equiv of picolinium **4e** to give compound **6a**. The latter was condensed with 1 equiv of indolium **1b** to afford dye **6**.

The optical properties and the pK_as of the prepared dyes are summarized in Table 1. All dyes, with the exception of compound **3**, exhibited fluorescence emission in the NIR region. This observation suggested that these dyes have the ability to form a new donor-two-acceptor fluorochrome after the phenolic species is converted into a phenolate. Dye **1** absorbance and fluorescence spectra are presented in Figure 9 (the other dyes spectra can be found in the Supplementary data). To demonstrate the turn-ON option of the probe, the spectra were measured under two pH conditions. For evaluation of the protonated form, the measurement was taken at pH lower than the pK_a of the phenol; for analysis of the deprotonated form, the measurement was taken at pH higher than the pK_a. As expected, a large red-shift was observed in absorption spectrum when the spectra of the protonated (λ_{max} = 410 nm) and the deprotonated (λ_{max} = 660 nm) forms were compared. Importantly, strong fluorescence emission was obtained in the NIR region for the protonated form, whereas no such fluorescence was observed for the deprotonated form. Dye **1** exhibited the

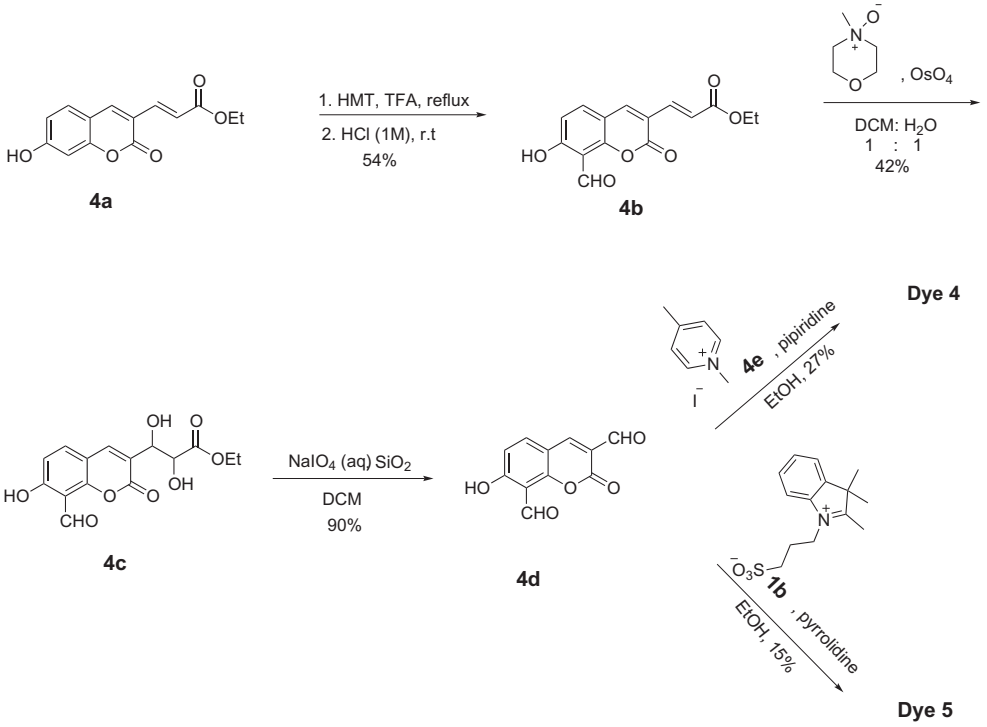


Figure 7. Chemical synthesis of dyes **4** and **5**.

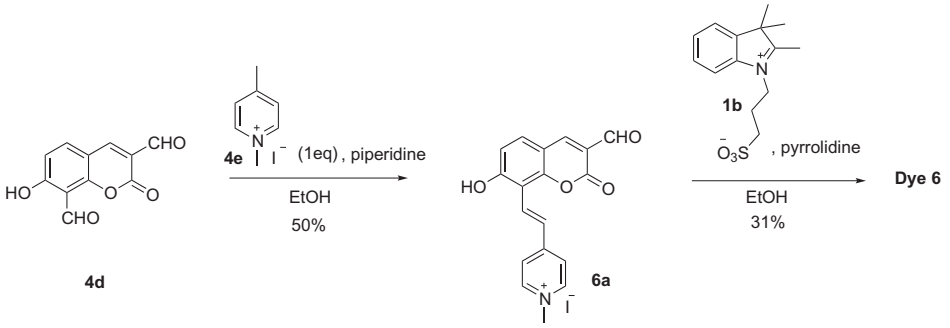
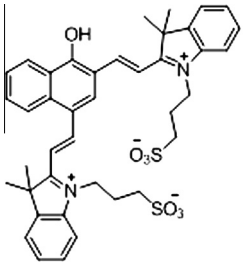
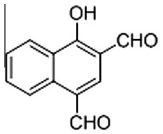
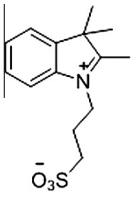
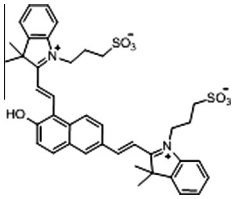
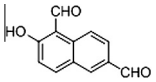
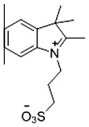


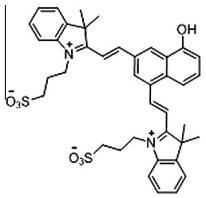
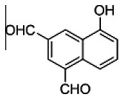
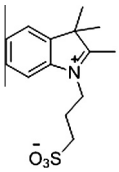
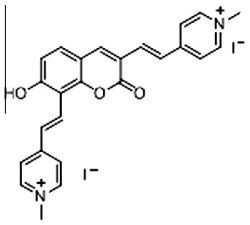
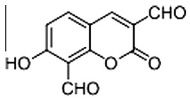
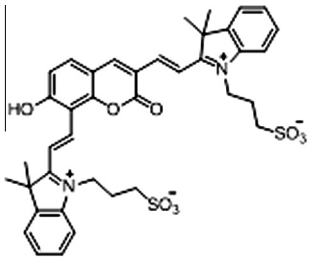
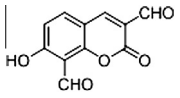
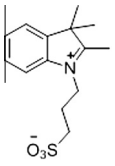
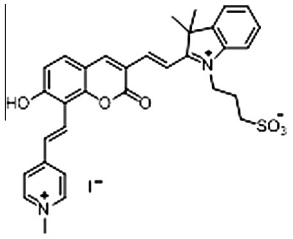
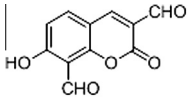
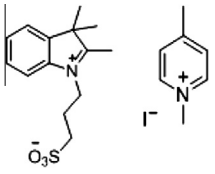
Figure 8. Chemical synthesis of dye **6**.

Table 1
Chemical structures and spectroscopic data of donor-two-acceptor based fluorogenic dyes obtained from naphthol or hydroxycoumarin donors

	Dye structure	Donor	Acceptors	λ_{ex}	λ_{em}	ε	Φ (%)	pK_a
1				510, 660	740	46,046	7.2	1.95
2				480, 620	750	26,472	1.4	4.25

(*continued on next page*)

Table 1 (*continued*)

	Dye structure	Donor	Acceptors	λ_{ex}	λ_{em}	ε	Φ (%)	pK_a
3				400, 570	–	17,391	–	7.65
4				450, 500	650	22,714	5.5	5.43
5				480, 600	700	28,074	16.7	3.8
6				450, 570	670	39,352	12.8	4.42

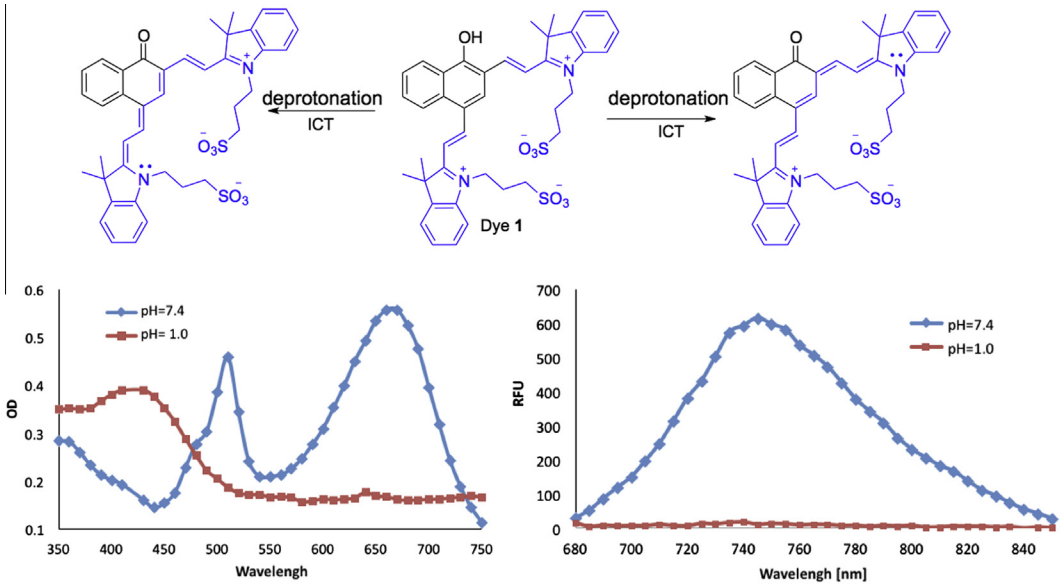


Figure 9. (Left) Absorption spectra of probe **1**, 25 μM in PBS pH 7.4 (blue) and in PBS pH 1.0 (red). (Right) Fluorescence spectra of probe **1**, 25 μM in PBS pH 7.4 (blue) and in PBS pH 1.0 (red), λ_{ex} 670 nm, λ_{em} 740 nm.

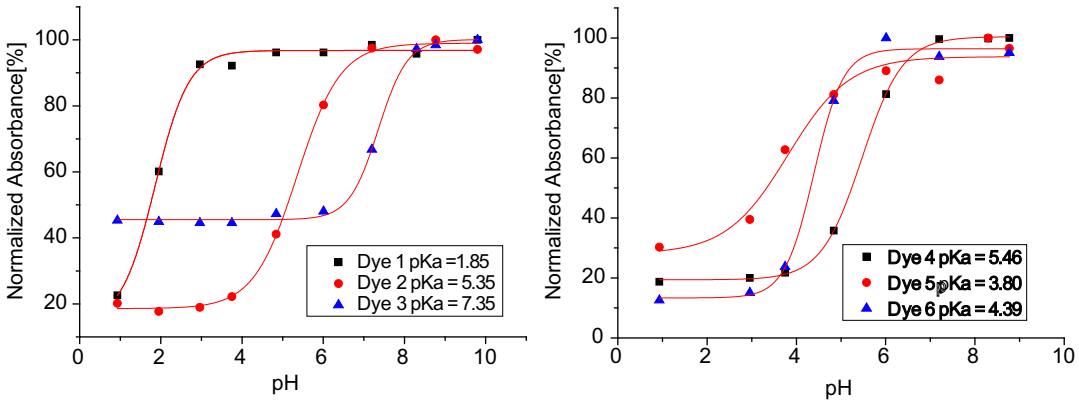


Figure 10. Determination of pK_a values for the phenols shown in Table 1.

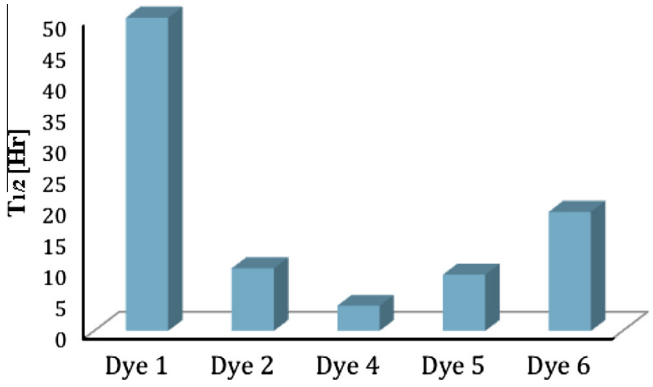


Figure 11. Fluorescence stability expressed as the half-life of the indicated dyes in PBS (pH 7.4) at 37 °C. Each dye was excited at the wavelength given in Table 1.

longest excitation/emission wavelength-pair of the dyes prepared based on the donor-two-acceptor design.

The relatively large shift in absorption spectrum between protonated and the deprotonated forms of these dyes allows convenient measurement of the pK_a values of the dyes. The pK_as of the six dyes were measured by monitoring the λ_{max} of their UV–vis absorbance as a function of the environmental pH (Fig. 10). The relative low pK_a values obtained for the phenolic dyes indicate that under physiological conditions, these dyes are in the phenolate form. Under typical intracellular conditions, the intramolecular-charge-transfer mode of action will occur, and the dyes will emit NIR fluorescence.

In order to assess which of dyes is most suitable for in vitro and in vivo imaging, we evaluated their fluorescence stability under aqueous conditions. The dyes were incubated in PBS (pH 7.4) at 37 °C, and their NIR fluorescent emission was monitored as a function of time (Fig. 11). Dye **1** had the highest stability under these conditions of the five dyes evaluated with a $T_{1/2}$ of over 50 h.

To demonstrate the potential of these dyes to serve as imaging reagents, we synthesized a probe for mitochondrial imaging. The synthesis was achieved as outlined in Figure 12. Indolium-acid **7b** was activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and hydroxybenzotriazole followed by the addition of compound **7a** to give indolium-amide **7c**. Two equivalents of this indolium were condensed with dialdehyde **1a** to afford probe **7**.

This probe is a derivative of dye **1** linked to a triphenylphosphonium moiety, which is known to be internalized by the mitochondria of cells.[18] The delocalized positive charge on the lipophilic triphenylphosphonium cations promotes their mitochondrial membrane potential dependent accumulation into mitochondria and their direct passage through phospholipid bilayers. Consequently, these molecules are useful in a range of mitochondrial studies. DA-3 mammary adenocarcinoma cells were incubated with the probe and imaged by confocal microscopy. The image in Figure 13 clearly shows that the mitochondria of these cells are fluorescent. The design of an analogue with a masked phenol that will be removed under oxidative stress could be a useful extension of this example.

The described dye molecules are composed of a phenol latent donor conjugated with two acceptors. The latent donor is turned ON upon formation of a phenolate ion. This unique design enables an intramolecular charge transfer from the activated donor to one

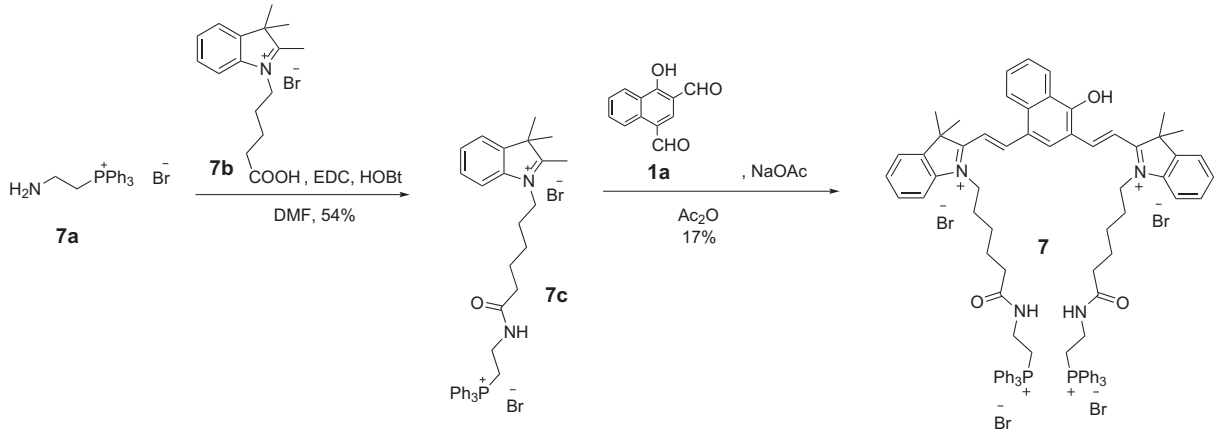


Figure 12. Chemical synthesis of mitochondrial imaging probe **7**.

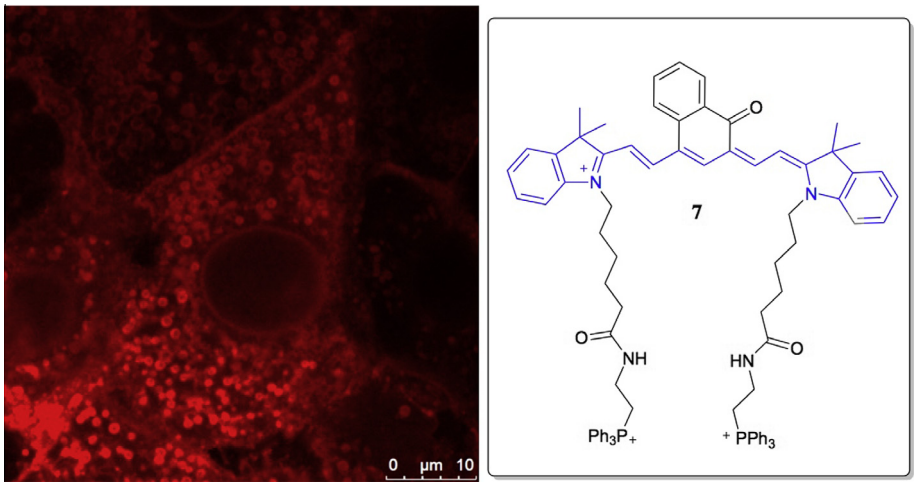


Figure 13. Mitochondrial imaging using probe **7** in cultured mouse DA-3 mammary adenocarcinoma cells (ex/em, 635 nm/710 nm).

of the two acceptors to form a new push–pull conjugated chromophore between the former two acceptors. The newly formed push–pull structural element has a larger π-system than that of the original donor–acceptor and thus can emit fluorescence at longer wavelengths. The introduction of naphthol or of hydroxycoumarin as the donor moiety has extended our repertoire of fluorogenic probes. Five of the six new dyes demonstrated good optical characteristics with fluorescence emission in the NIR region. Interestingly dye **3** was the only one that failed to fluoresce, probably, due to the absence of an acceptor moiety on the phenolic ring. Indeed phenol **3** had the highest pK_a value among the prepared dyes, and although a visible red-shift was observed in the deprotonated form, no fluorescence was obtained.

3. Conclusions

In summary, we have introduced a new repertoire of fluorogenic dyes through our strategy for long-wavelength fluorogenic probes. The design is based on a donor-two-acceptor π-electron system that can undergo an intramolecular charge transfer to form a new fluorochrome with a longer π-conjugated system. Two different latent donors, naphthol and hydroxycoumarin were conjugated with indolium or picolinium acceptors into the modular probe platform to generate versatile dye compounds. One of the dyes, which had significant stability at physiological pH in aqueous conditions, was conjugated with a lipophilic cation that is readily taken up by mitochondria. Confocal images of DA-3 mammary adenocarcinoma cells incubated with this probe indicated that the probe penetrates cells and can be used as mitochondrial imaging reagent. This unique strategy for dye design has opened a new doorway for further NIR fluorescence probe discovery.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.049.

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