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Synthesis of pH-Activatable Red Fluorescent BODIPY Dyes with Distinct Functionalities

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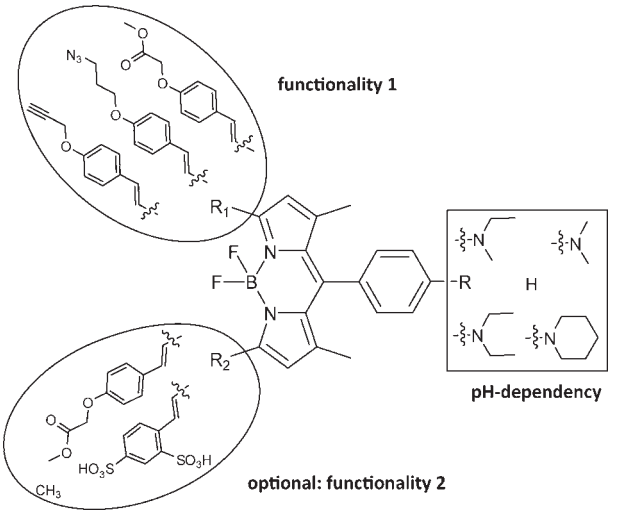
ABSTRACT



A series of tunable pH-dependent BODIPY dyes were synthesized and further functionalized in a Knoevenagel condensation reaction with various aldehydes. In this fashion, monofunctional dyes containing an alkyne, azide, or carboxylic acid (masked as its methyl ester) as ligation sites as well as asymmetrical bifunctional dyes were obtained, without compromising their pH-dependency. In addition, fluorescence excitation and emission maxima for these dyes were shown to be significantly red-shifted in comparison to their tetramethyl precursors.

In recent years fluorescent dyes have found widespread use in chemical biology applications. An important class of dyes are those based on the 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (BODIPY) scaffold. BODIPY dyes usually show excellent photochemical properties, such as high molar absorption coefficients, high quantum yields, and narrow emission bandwidths. Moreover, they are relatively stable under physiological conditions.[1]

Synthetic efforts have been directed toward the formation of analyte-sensitive dyes, and numerous reports can be found in the literature.[1c] Particularly useful examples are the pH-sensitive BODIPY dyes that are responsive to protonation.[2] Intracellular pH is an important factor in many physiological processes, and by using a pH-activatable dye, selective visualization of processes in acidic cellular compartments becomes possible. pH-sensitivity

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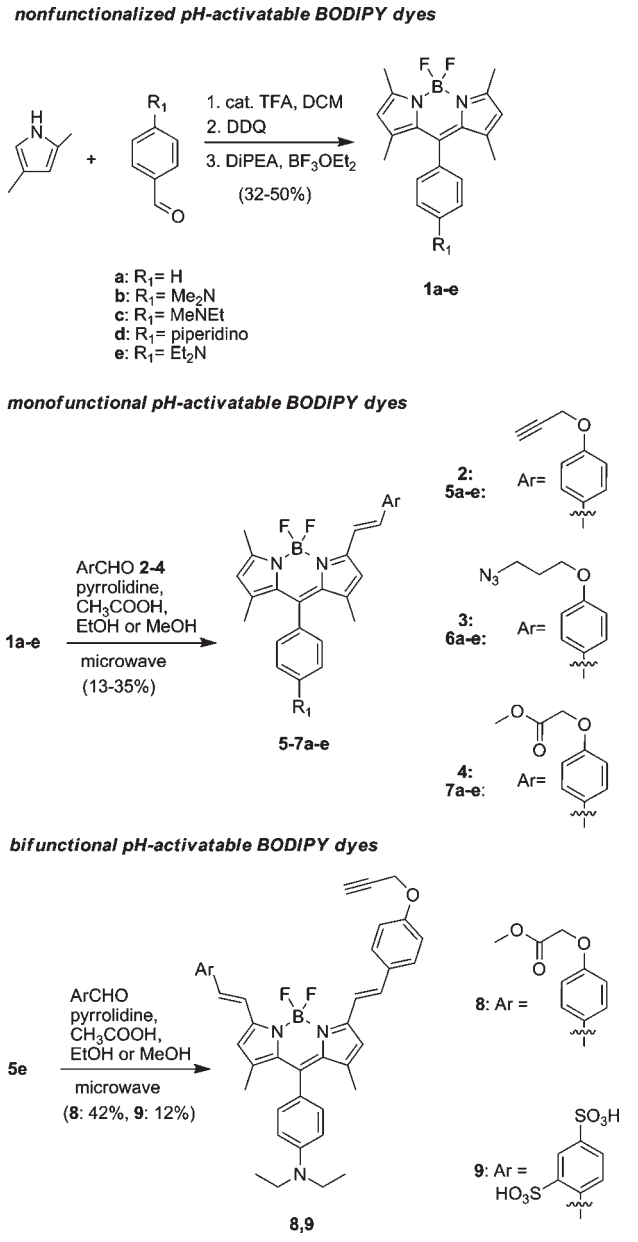
Scheme 1. Synthesis of Nonfunctionalized, Monofunctional, and Bifunctional BODIPY Dyes



is usually accomplished by incorporation of a *p*-(*N*,*N*-dialkyl)aniline moiety at the *meso*-position of the BODIPY core.[3−5,5,6,7a] Due to photoinduced electron transfer (PeT)[7] fluorescence is quenched at neutral or basic pH. Upon protonation of the aniline nitrogen fluorescence intensity increases drastically.

In a recent paper by Urano et al., the development of a series of pH-activatable dyes with varying pK_a values is described.[4] These can be easily transformed into bifunctional dyes, but for monoconjugation they are less convenient due to their symmetry.[5] Other groups have investigated the tetramethyl-BODIPY scaffold with a *meso*-aniline substituent.[3,7a,8] The methyl groups adjacent to the nitrogens are susceptible to a Knoevenagel-type condensation with benzaldehydes, yielding dyes with red-shifted absorption and emission maxima.[2b,c,e,9−11] The potential of the Knoevenagel reaction to extend the conjugated system is widely acknowledged, and the reaction has been used to construct large libraries of (pH-independent) BODIPY dyes.[12] To our knowledge, use of the Knoevenagel reaction to incorporate ligation handles in the BODIPY dye has not been employed, although that would allow covalent attachment to other (bioactive) molecules, which is vital for applications in chemical biology. Often used ligation reactions comprise the Staudinger−Bertozzi ligation (involving azides),[13] the copper(I) catalyzed Huisgen 1,3-cycloaddition (involving azides or alkynes),[14] and, in a more traditional fashion, amide/ester formation (involving carboxylic acids). We here show that by using different *N*,*N*-alkylated aniline derived tetramethyl-BODIPYs as a scaffold in combination with functionalized (azide, alkyne, methyl ester) benzaldehydes for the Knoevenagel reaction, a series of tunable pH-activatable dyes can be prepared that have the further advantage of being red-shifted compared to the currently known pH-dependent BODIPYs. In addition, we reveal that by applying the Knoevenagel reaction twice, the synthesis of asymmetric bifunctional dyes with either an extra conjugation handle or two sulfonic acids to increase the water solubility can be achieved.

Starting from commercially available *N*,*N*-dialkylamino benzaldehydes (or benzaldehyde in case of **1a**, the "always on" dye) and 2,4-dimethylpyrrole synthesis of nonfunctionalized BODIPY dyes **1a**−**e** proved straightforward with

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yields ranging between 32 and 50% (Scheme 1).[7a,15] To obtain monofunctional BODIPY dyes **5**–**7a**–**e**, three benzaldehydes were synthesized, containing either an alkyne (**2**),[16] azide (**3**),[17] or a methyl ester (**4**)[18] as the functional group. The use of a *tert*-butyl ester has been reported in a related study,[9c] however, literature precedence shows that methyl esters can be saponified using (mild) basic conditions while leaving the BODIPY core intact.[4,5,19] Subsequently, these benzaldehydes were used in a Knoevenagel condensation reaction with BODIPYs **1a**–**e**. Reaction times using classical Knoevenagel conditions (refluxing in toluene in the presence of piperidine/acetic acid) can be extensive (12–24 h).[2b,c,9c] Recent reports show that the use of microwave irradation considerably shortens reaction times (5–20 min).[12b,20] We therefore decided to also make use of microwave conditions, to synthesize various BODIPYs in a relatively short period of time. In case of benzaldehyde **4**, the reaction solvent was changed from ethanol to methanol to prevent transesterification of the methyl ester. Typically, reaction yields of the Knoevenagel reaction are low,[2b,9,20] and although our case forms no exception, the envisaged 15 monofunctional BODIPYs **5**–**7a**–**e** were all readily obtained. Importantly, a significant amount (30–50%) of unreacted starting material could easily be recovered by silica column chromatography.

BODIPY dyes **1a**–**e** contain two reactive methyl groups (at the 3- and 5-positions), and the main byproduct observed in the reaction was indeed the symmetrical distyryl substituted BODIPY. Under these conditions, we did not observe any formation of tetrastyryl substituted BODIPY dyes.[10a,11] We decided to make use of the acidity of the second methyl group and subjected monofunctional dye **5e** to another cycle of Knoevenagel condensation with benzaldehyde **4** to obtain bifunctional BODIPY dye **8**, containing two orthogonal ligation handles. As an alternative strategy we used the second methyl group to alter the solubility properties of the dye. BODIPY dyes tend to be fairly lipophilic and poorly water-soluble, which can be dimished by introduction of polar moieties such as sulfonic acids[23] or polyethylene glycol.[10] By introduction of a disulfonic acid containing styryl moiety *via* the Knoevenagel reaction water-soluble BODIPY dye **9** was synthesized in 12% yield. The low yield of this reaction was mostly due to extensive purification procedures to separate the product from pyrrolidinium acetate.

Table 1. Spectroscopic Properties Measured in TFA (135 mM)/Methanol

	λ_{abs} (nm)	λ_{em} (nm)	Φ_f[b,c]	pK_d[e]
1a	497	510	0.63[b]	–
1b	500	514	0.35[b]	4.49[a]/2.14
1c	500	513	0.44[b]	5.10[a]/2.95
1d	500	514	0.40[b]	5.06[a]/3.97
1e	500	513	0.56[b]	5.81[a]/4.05
5a	563	574	1.09[c]	–
5b	567	582	0.66[c]	2.13
5c	569	582	0.84[c]	2.93
5d	568	582	0.85[c]	3.92
5e	568	582	0.84[c]	4.07
6a	564	578	1.01[c]	–
6b	568	586	0.59[c]	2.08
6c	568	586	0.69[c]	2.93
6d	570	586	0.81[c]	3.91
6e	570	587	0.82[c]	4.05
7a	562	574	1.10[c]	–
7b	566	580	0.67[c]	2.08
7c	567	581	0.78[c]	2.96
7d	567	581	0.87[c]	3.94
7e	567	581	0.87[c]	4.10
8	640	655	–[d]	4.10
9	643[a]	660[a]	–[d]	5.81[a]

[a] Measured in citric acid/phosphate buffer. [b] Relative quantum yield (±0.1) with fluorescein (Φ_f = 0.925 ± 0.015 in 0.1 M NaOH)[21] as standard. [c] Relative quantum yield (±0.1) with rhodamine 101 (Φ_f = 1.00 ± 0.02 in ethanol)[22] as standard. [d] No standard available. [e] Apparent dissociation constant determined from curves of fluorescence vs p[TFA] (in MeOH) or pH (pK_a = −log K_d)

Evaluation of the spectroscopic properties of the dyes (Table 1) revealed small Stokes shifts, characteristic of BODIPY dyes, and moderate to excellent relative quantum yields. The fluorescence absorption and emission maxima of the dyes were shifted approximately 70 nm toward the red end of the spectrum with each Knoevenagel reaction (Table 1, Figure 1a). For biological applications such as live-cell fluorescence microscopy this is a great advantage, since levels of autofluorescence are lower at the red end of the spectrum. Besides, as a result of their narrow absorbance and emission peaks, the bifunctional dyes are compatible for use in the same experiment as other pH-dependent dyes.

To assess whether modifications of the BODIPY core by one or two Knoevenagel reactions would lead to any change in pH-dependency, the fluorescence intensities at different pH-values were measured for all pH-activatable fluorophores (Figure 1b–d; see also Supporting

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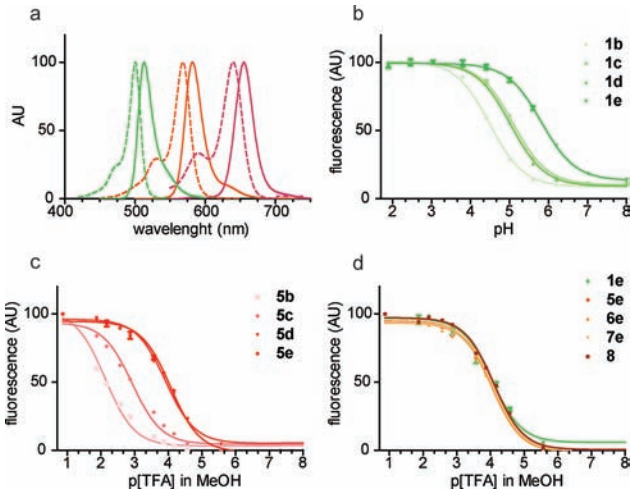

Figure 1. (a) Absorbance (- - -) and emission (—) spectra of compounds **1a**–**e** (green), **5**–**7a**–**e** (orange) and **8,9** (purple) in TFA/methanol. (b) Fluorescence intensity vs pH curves for compounds **1b**–**e**. (c, d) Fluorescence intensity vs p[TFA] curves for compounds **5b**–**e** and for nonfunctionalized (**1e**), monofunctional (**5**–**7e**), and bifunctional (**8**) diethylaniline derivatives.

Information, Figure S1). Since the water solubility of dyes **5**–**8** was low, their fluorescence was determined as a function of the concentration of trifluoroacetic acid (TFA) in methanol. The apparent pK_a (in aq. buffer) or pK_d (in methanol/TFA)[1c] of the dyes decreases in the order diethyl > piperidino > ethylmethyl > dimethyl (Figure 1b,c, Table 1), which corresponds to the electron-donating properties of the substituted amines and the trend found for previously reported pH-dependent dyes.[4,5,8] No change in pH-dependency was observed for the various mono- and bifunctional dyes compared to their nonfunctionalized counterparts (Figure 1d, Table 1). Hence, we expect that attachment of these dyes *via* their ligation handle to (bio)active molecules will result in constructs with the same advantageous fluorescence characteristics as the original dyes.

Finally, to examine the stability of the dyes when used in a ligation reaction, we used dye **5e** in a traditional copper(I) catalyzed Huisgen 1,3-cycloaddition[14] with the azide-modified proteasome inhibitor epoxomicin.[24] This resulted in the pH-dependent BODIPY-epoxomicin **10** in good yield (Figure 2). Current efforts are directed toward the ligation of these dyes to other bioactive molecules.

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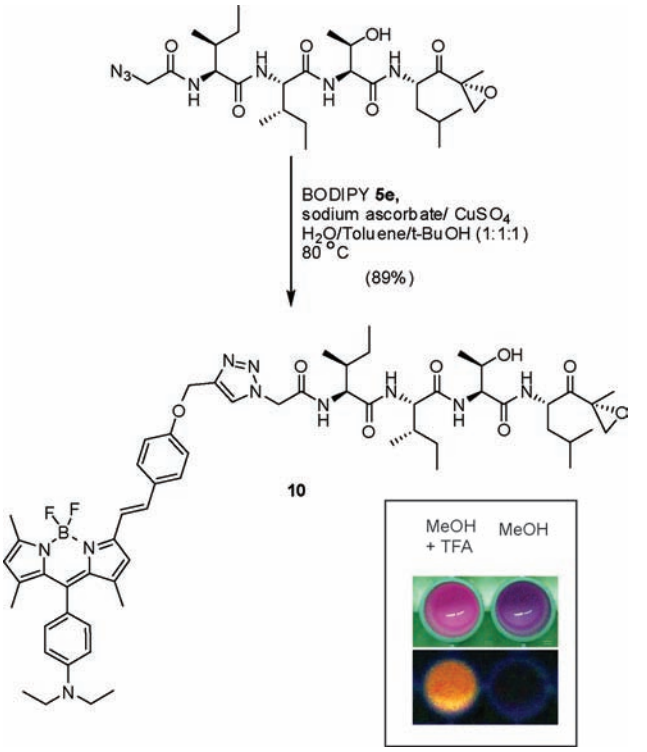

Figure 2. Synthesis of BODIPY-epoxomicin. Inset: pH-dependent fluorescence of compound **10**.

In summary, we have synthesized a series of tunable pH-dependent BODIPY dyes that contain a (bio)conjugation handle for incorporation in larger constructs. We envisage that these dyes will find their application in diverse areas of chemical biology such as activity-based protein profiling and proteomics. In particular, we feel that these pH-activatable dyes are ideally suited for use in live-cell fluorescence microscopy experiments as noninvasive tools to selectively image cellular compartments of interest, based on their acidity. Because of the generality of the approach, the methodology could in the future be extended toward the development of many other (analyte-sensitive) BODIPY dyes containing the functionality or property of interest.

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Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.