

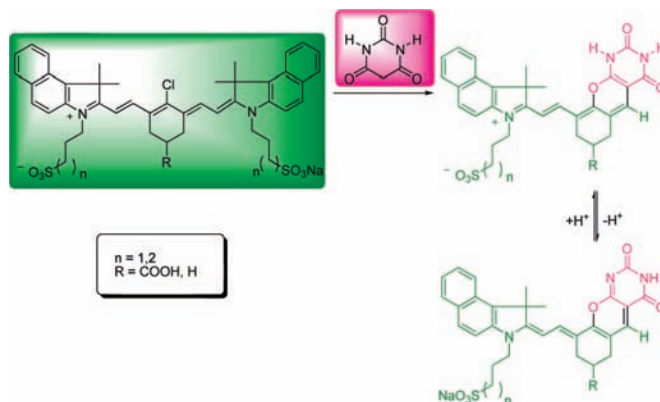
## Near-Infrared Fluorescent pH-Sensitive Probes via Unexpected Barbituric Acid Mediated Synthesis

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## ABSTRACT



Novel near-infrared pyrimidine-fused pH fluorescent probes were prepared by an unusual barbiturate-mediated debenzoindolization and subsequent heteroannulation. A plausible mechanistic pathway is proposed, and the final structures were further elucidated by 2D-NMR. All new compounds are highly fluorescent in the near-infrared region and possess excellent spectral sensitivities to environmental pH changes.

Near-infrared (NIR) cyanine dyes are increasingly employed as fluorescent probes for interrogating biological processes such as enzyme activity,<sup>1</sup> nitric oxide,<sup>2</sup> calcium,<sup>3,4</sup> zinc,<sup>5</sup> and proton concentration.<sup>6,7</sup> The key idea is to incorporate different functionalities into a cyanine molecular framework

to obtain environment-sensitive fluorophores where the spectral properties are influenced by the presence of specific compounds or ions in complex samples. This requires a precise choice of functionalities as well as a feasible synthetic methodology. A majority of these dyes are constructed via a well-established S<sub>N</sub>1 mechanistic pathway where the *meso*-chlorine atom of heptamethine dye is substituted with various nucleofugal functionalities.<sup>8</sup> This route renders an array of fluorophores that contain highly functional aminoalkyl,<sup>5,9</sup> hydroxyalkyl,<sup>10</sup> hydroxyaryl,<sup>11</sup> thioalkyl,<sup>12</sup> and thioaryl<sup>13</sup> substituents that can be further conjugated to biomolecules

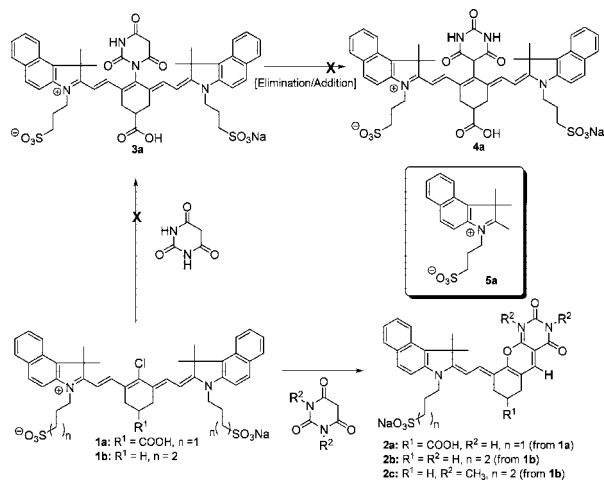
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or other metal ligands. Recently, we reported the first C–C bond formation by the Suzuki-coupling approach at the *meso*-position to give functionalized aryl-substituted fluorophores with reinforced chemical stability.<sup>14</sup>

In this paper, we report an unusual barbiturate-mediated synthesis of NIR fluorescent pH-sensitive probes.

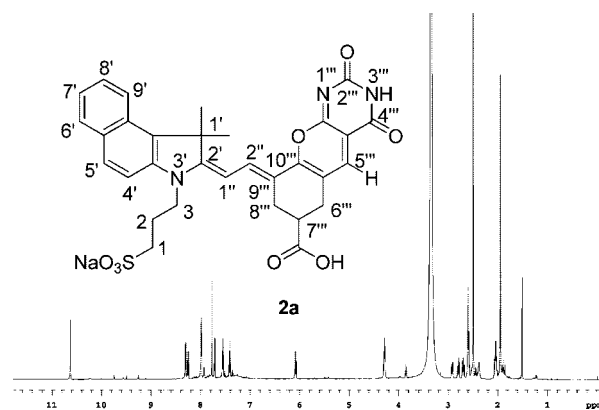
The new probes were prepared by a “one-pot” synthetic procedure (Scheme 1). Previously, Nagao et al. utilized

**Scheme 1.** Synthesis of Pyrimidine-Fused Dyes **2a–2c**



barbituric acid and substituted chlorine atom at the *meso*-position of polymethine dye (IR-820) by heating at 50 °C in a 1:1 mixture of MeOH/ $\text{CH}_2\text{Cl}_2$  to give the resultant dye with an absorption maxima at 795 nm with no apparent pH-responding properties.<sup>15</sup> Surprisingly, reaction of cyanine dye **1a** with barbituric acid in acetonitrile in the presence of TEA at room temperature resulted in the formation of a blue solid with absorption maxima at 690 nm and a trace amount of dye with absorption maxima at 795 nm. Initially, the dye structure with absorption maxima at 690 nm was tentatively assigned as **3a** as shown in Scheme 1. This assignment was based on the previous findings that *N*-methyl substitution at the *meso*-position resulted in the hypsochromic shift of approximately 200 nm from the parent dye (820 nm).<sup>8</sup> On the basis of these results, it was hypothesized that **3a** is first formed as a kinetic product which then subsequently converted to dye **4a** via the elimination/substitution process. To validate this hypothesis, dye **3a** was isolated in pure form and further treated with barbituric acid under the same reaction conditions. However, the reaction failed to produce **4a** from **3a**. During the course of the

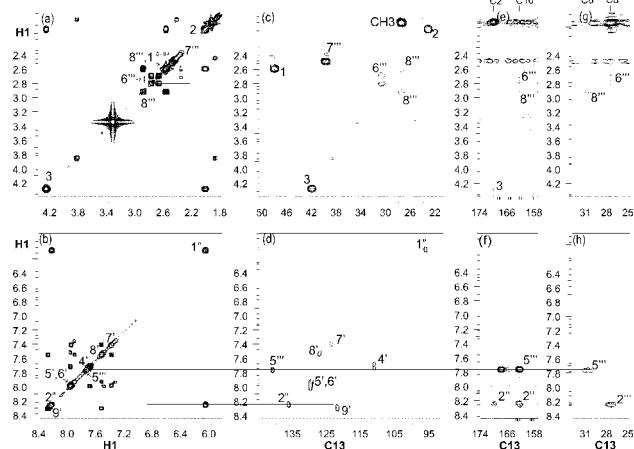
investigation, it was also detected that the LC-MS spectrum of the reaction solution exhibited an intense peak at  $m/z$  331 corresponding to (**5a**)<sup>+</sup>. However, the LC-MS analysis of the starting chloro dye **1a** did not exhibit the same spectral pattern, suggesting that it is not a residue from the starting dye but rather a reaction byproduct. Moreover, the tentatively assigned structure **3a** did not coincide with the LC-MS and NMR data. On the basis of the LC-MS and  $^1\text{H}$  NMR analysis (1D and 2D), the final structure was reassigned as **2a**. The  $^1\text{H}$  NMR spectrum exhibits two distinct singlet resonances at 7.77 ppm corresponding to H-5''' and 10.62 ppm corresponding to the –NH proton as shown in Figure 1.



**Figure 1.** Structure and  $^1\text{H}$  NMR spectrum of **2a**.

The final structure of **2a** was then further confirmed by extensive NMR experiments (COSY, NOESY, HMQC). Proton and carbon chemical shifts of **2a** were assigned by analysis of COSY and HMQC and HMBC spectra. Resonances of the indole N-substituents (H1–H3), vinyl bridge (H1''–H2''), and H6'''–H8''' chromeno fragment were identified by the through bond spin propagation. The correlated proton resonances at 4.29 and 8.25 ppm (Figure 2a,b) were unambiguously assigned to H3 and H2'', respectively. Proton connectivity was confirmed by the vicinal J-coupling in COSY experiments. All the connections between adjacent protons were observed, and a continuous path indicates the segment of H1'' to H2'' at the vinyl bridge as well as the H1–H3 (Figure 2a, 2b) methylene chain in indole N-substituents. Assignments of **2a** were obtained by tracing the HMQC and HMBC cross peaks to the same proton chemical shift at the COSY spectra. Connections between Figures 2a, 2c and 2b, 2d revealed that not only all aliphatic and aromatic carbon–carbon connections were observed but also the methylene H6''' and H8''' in **2a** can be assigned unambiguously. In particular, the observed simultaneous HMBC correlations of C10''' to 6''', 8''', 5''', and 2'' protons (Figure 2e, 2f) confirm the connection of the vinyl bridge to the chromeno-pyrimidine side. Furthermore, correlations of H5''' to C10''', C6''', and cross peaks of H2'' to C10''', C8''' in Figure 2f, 2h revealed that the vinyl bridge is connected through the C9'''–C10''' double bond of the chromeno

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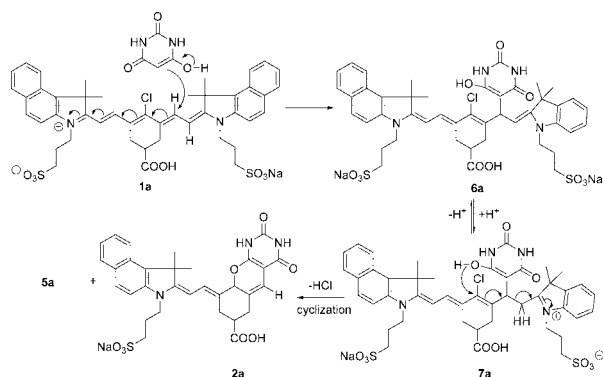


**Figure 2.** Expansion of (a) (b) COSY, (c) (d) HMQC, and (e) (f) (g) (h) HMBC of 600 MHz spectra of **2a** in DMSO- $d_6$  at 25 °C. Connections for one-bond and long-range proton–carbon correlations are indicated.

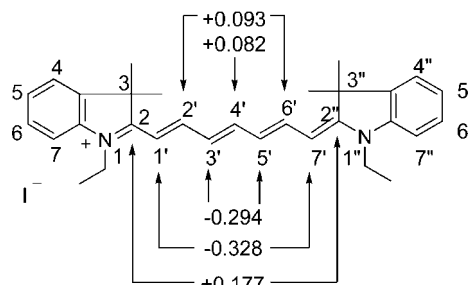
segment. Likewise, multiple bond carbon–proton correlations of C2' to 2'', 3, and 1'-methyl protons confirm the vinyl bridge–indole connections.

A plausible mechanistic pathway proposed for the synthesis of pyrimidine derivative **2a** is shown in Scheme 2.

**Scheme 2.** Plausible Mechanistic Pathway



Addition of a barbituric acid moiety at the C-2'(6') position of heptamethine dye and subsequent enamine–imine equilibrium leads to cyclization/elimination to give the final compound **2a** and the byproduct **5a**. While nucleophilic addition at the C-2(2'') or C-4' position of cyanine dye is known, the addition at the C-2'(6') has not been reported (Figure 3). The ground-state electron density of a similar heptamethine cyanine system can provide insight into the chemical reactivity of cyanine dyes. As shown in Figure 3, the *meso* (C4') carbon of the heptamethine chain was found to possess the lowest magnitude of positive character (+0.082), while the C1' and C7' accommodate the greatest amount of negative charge (−0.328). Accordingly, the susceptibility to nucleophilic addition at the carbons is in the decreasing order of C2(2'') > C2'(6') > C4'.<sup>16</sup>

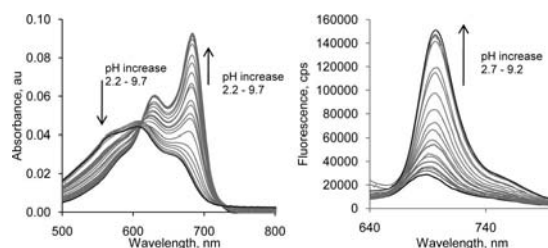


**Figure 3.** Point charges of a selected indolium heptamethine cyanine dye at selected carbon atoms obtained from ampac calculations.<sup>16</sup>

This trend is more evident in the presence of a base where the reversible addition of OH<sup>−</sup> occurs at the C-2 position.<sup>17</sup> However, in the presence of a bulky nucleophile such as barbituric acid, the sterically hindered C-2 position facilitates the competitive addition at C-2'(6') or C-4' positions. In addition to the steric factor, the solvent may also play a major role in the mechanistic pathway and the resultant outcome of the reaction.

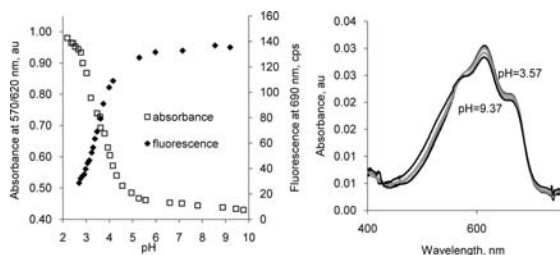
The scope of the reaction was then further exploited utilizing a commercially available dye **1b** (IR-820) with barbituric acid or *N,N*-dimethylbarbituric acid under identical reaction conditions to give dye **2b** or **2c**, respectively. NMR analysis of **2b** exhibited similar carbon–proton correlations as in **2a** except that the H7''' and C7''' were shifted upfield (Figure S7 in the Supporting Information) due to the absence of a carboxyl group. Although the H6''' and H8''' resonances were overlapped, however, the C6''' and C8''' can be distinguished by the HMBC correlations to H5''' and H2'', respectively.

Spectrophotometric analysis of **2a** at various pHs revealed that both absorption and fluorescence emission are pH dependent. The absorption spectra of **2a** exhibit an intense peak at 690 nm in neutral pH with a hypsochromic shift to 605 nm at acidic pH (Figure 4, left). The fluorescence



**Figure 4.** Absorption (left) and emission spectra (right) of **2a** in water, excitation 605 nm.

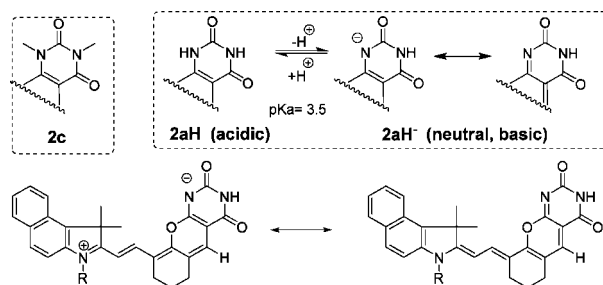
intensity was minimal below pH 2 but increased >6-fold as pH increased from 2 to 9 (Figure 4, right). The  $pK_a$  values of **2a** determined by absorption and fluorescence titration plots (Figure 5, left) were found to be comparable at  $\sim 3.5$ , suggesting similar acidity in the ground and excited states.



**Figure 5.** Left: Titration absorption and emission intensity diagrams of **2a** in water, excitation 605 nm. From absorbance:  $pK_a = 3.58 \pm 0.00$ . From emission:  $pK_a = 3.53 \pm 0.02$ . Right: Absorption spectra for dimethylated **2c** show no change between pH 3.57 and 9.37.

A drastically low  $pK_a$  ( $\sim 3.5$ ) for N–H dissociation equilibrium of **2a** compared to the typical  $pK_a$  of  $\sim 9.5$  for uracile derivatives is attributed to the presence of delocalized positive charge across the cyanine molecular framework in which deprotonation results in the loss of net charge. This process is energetically favorable and thus lowers the  $pK_a$  values. Deprotonation of one of the two nitrogens with the formation of uracilate and its resonant form is shown in Figure 6. Compound **2b** showed a similar pH response as **2a** (Figure S8 in the Supporting Information).

The proposed isomeric forms are more evident when compared to the optical properties of *N,N*-dimethylated barbituric derivative **2c**. The absorption spectra of **2c** in the absence of a deprotonation site did not exhibit spectral sensitivities to various pHs and showed the absorption maxima at 605 nm at pH ranges from 2–9. The optical properties, including the absorption and emission spectra, as well as the quantum yield of **2c** are almost identical to those of **2aH** (acidic), suggesting that both **2c** and **2aH** have similar electronic distribution. Thus, it is evident from these studies that the presence of a free –NH moiety is crucial in



**Figure 6.** Top: Protonated and deprotonated forms of **2a** from comparative analysis with **2c** under acidic and basic conditions. Bottom: resonance between two forms of **2a** under neutral and basic conditions.

inducing pH sensitivity where substitution of N–H to N–CH<sub>3</sub> did not facilitate the spectral changes as a function of pH.

In conclusion, we demonstrated an unusual barbiturate-mediated approach to NIR fluorescent pH probes. The structural features of newly developed fluorescent pH probes suggest a novel substitution/elimination pathway. The detailed mechanistic study and its application are underway in our laboratory and will be reported in the future.

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**Supporting Information Available:** Synthesis and characterization (<sup>1</sup>H NMR, <sup>13</sup>C NMR, <sup>1</sup>H–<sup>1</sup>H COSY, HMQC, HMBC spectra, and UV–vis and fluorescence spectra) of **2a–2c**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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