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A new Vilsmeier-type reaction for one-pot synthesis of pH sensitive fluorescent cyanine dyes

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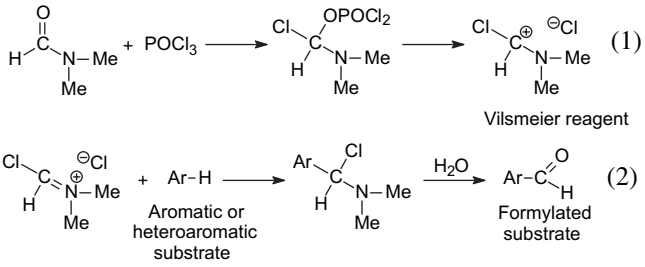
Abstract—A new Vilsmeier-type reaction is suggested for the synthesis of novel indocarbocyanine pH sensors, which are fluorescent when protonated but nonfluorescent upon proton abstraction. These sensors show significant ratiometric UV–visible as well as fluorescence spectral changes upon subtle variation of pHs with pK_a values near neutral.

1. Introduction

Cyanines exhibit large extinction coefficients ($\varepsilon>10^5$ $M^{-1}\,cm^{-1}$) and moderate fluorescence quantum yields, leading to widespread applications such as photosensitizers, stains, and fluorescent labels and sensors.[1] Chemical sensors are now recognized as a valid alternative to conventional instrumentation. This is especially true for analytical problems requiring on-site and real-time acquisition of data such as process control, environmental, and biomedical monitoring.[2] In particular, acid/base sensors capable of indicating the presence or absence of protons are of great importance akin to life sciences, detectors responding to environmental changes, and memories or logic gates in nanotechnology.[3–8] A fluorescence-based technique for pH measurement offers significant advantages over other techniques due to its generally nondestructive character, high sensitivity, and specificity. Numerous fluorescent systems displaying bimodal response on and off at near neutral pH 4.5–8 have been reported and have led to practical applications for intracellular pH measurements.[9] Cyanine dyes have been used as sensor molecules for the fluorescent detection of analytes in diagnostics.[10–12] Despite their success, only few examples of fluorescent cyanine biosensors sensitive to proton concentration have been reported.[11–13]

Since the first publication appeared in 1927, the Vilsmeier or Vilsmeier–Haack reaction has been applied to an immense variety of substrates, from substituted benzenes to complex heterocycles.[14] Vilsmeier reagent, an iminium salt with weak electrophilic character, results from the reaction between an acid chloride (e.g., $POCl_3$, $SOCl_2$, $(COCl)_2$, and $COCl_2$) and an amide, usually DMF. Further reaction of Vilsmeier reagent with a reactive aromatic substrate[15] followed by basic workup affords acylation products via electrophilic aromatic substitution.[14] If DMF is used in the reaction, the product of Vilsmeier reaction is an aldehyde, thus Vilsmeier reaction is often called as Vilsmeier formylation (Scheme 1).



Scheme 1. Vilsmeier–Haack reaction.[14]

Vilsmeier reaction has been utilized in the synthesis of asymmetric cyanines via formylation of Fischer's base followed by Knoevenagel condensation with the CH-acidic compound as electron acceptor.[16,17] During our investigations into the syntheses of pH sensitive cyanines, we envisioned the possibility of using a new type of Vilsmeier reaction in one-pot process to obtain the desired products. This route, if realized, would allow among other possibilities, an easy access to new pH sensitive cyanines containing sulfonic acid group that aids solubility in aqueous media and

Keywords: Fluorescent pH sensors; Vilsmeier reaction; Vilsmeier–Haack reaction; Cyanine dyes.
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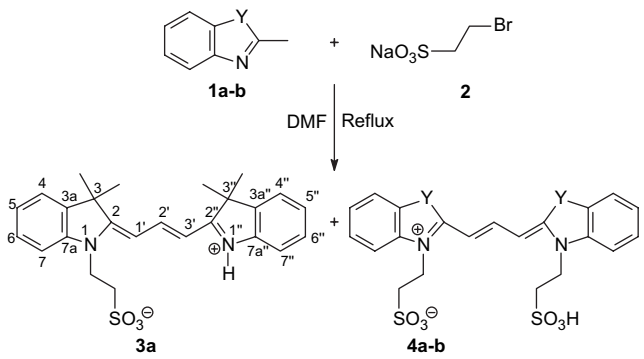
also reduces sensor aggregation in solution.[18] Herein, we wish to report the synthesis, reaction mechanism, and spectroscopic characterization of the new trimethine cyanine pH sensor dyes.

2. Results and discussion

2.1. Synthesis

Generally, the standard methods for preparation of cyanine dyes are based on the condensation of suitable heterocyclic salt with polymethine forming agent in the presence of base.[19] Similarly, pH sensor cyanine dyes are prepared from the condensation of heterocyclic salt with heterocyclic base and polymethine forming agent in the presence of acid and its anhydride together with pyridine.[11,13] The synthesis reported here involves a single step where the heterocyclic base **1a** was refluxed with excess of bromoethanesulfonate **2** in DMF to give the pH sensor dye **3a** in a mixture with the corresponding symmetric indocarbocyanine **4a** (Scheme 2). Purification of the crude product by silica gel chromatography using gradients of $MeOH/CH_2Cl_2$ afforded the first fraction **3a** as a red crystal in 32% yield followed by the second one **4a** as a red powder in 52% yield. On the other hand, the heterocyclic base **1b**, where Y=S did afford only the corresponding symmetric thiacarbocyanine **4b** as a violet crystal in 8% yield after being purified in a similar manner to **4a**.

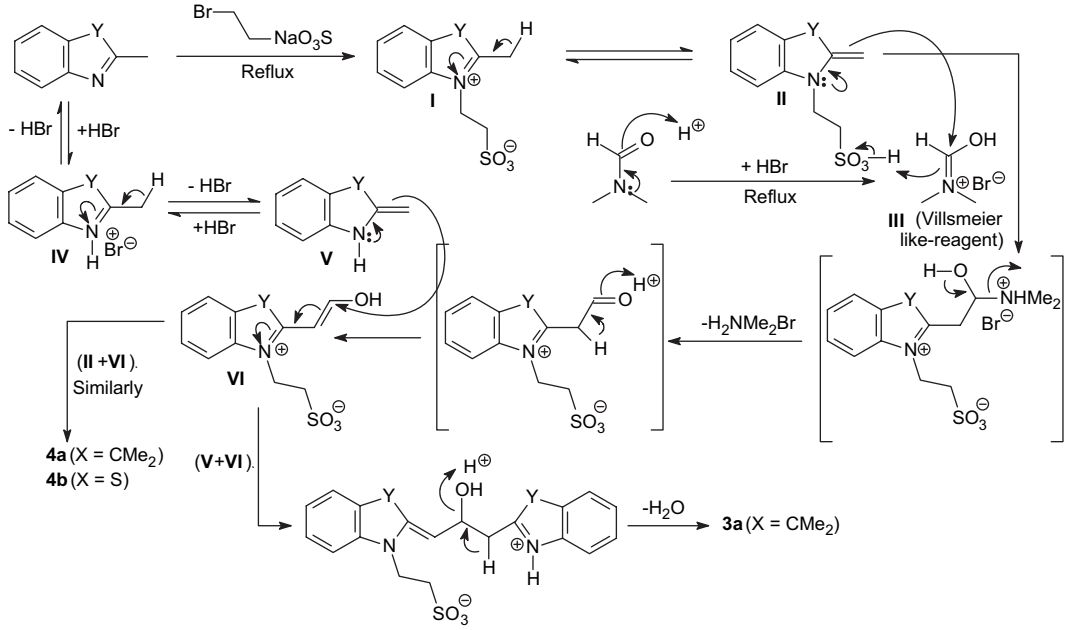


Scheme 2. One-pot synthesis of pH sensor dye **3a**.

The structures of the new dyes **3a**, **4a**,[20] and **4b** were evidenced by their HRMS, ^1H NMR, ^{13}C NMR, and IR data.

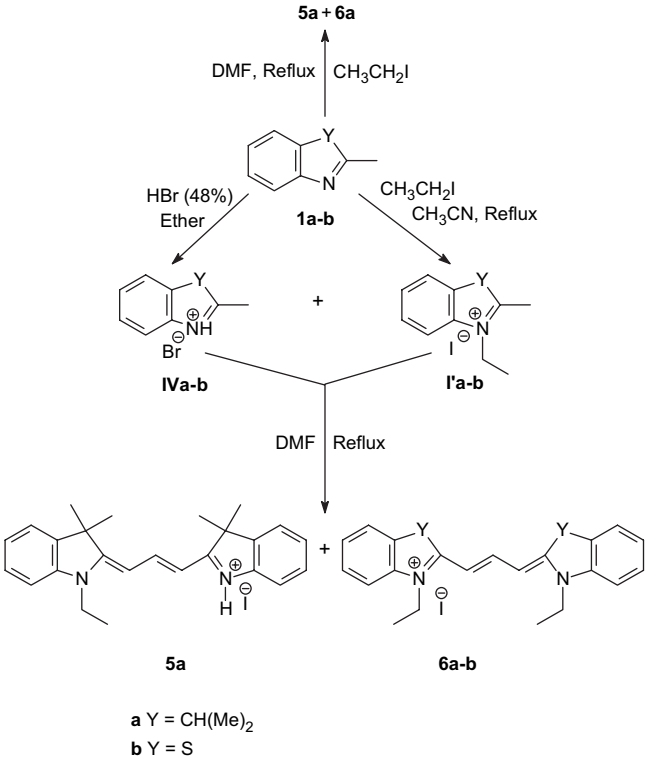
2.2. Proposed new Vilsmeier-type reaction

The formation of pH sensor dye **3a** as well as its corresponding symmetric indocarbocyanine one **4a** in one-pot synthesis could be explained by the mechanism proposed in Scheme 3. The reaction starts from the expected formation of the betaine salt **I** together with the protonated form **IV** and Vilsmeier-like reagent **III**, both formed by reflux in the presence of traces of hydrobromic acid generated in situ. Formylation of base **II** (obtained in situ from **I**) with Vilsmeier-like reagent **III** affords the enolated betaine form **VI** after elimination of dimethylammonium bromide. Condensation of **VI** with either **V** or **II** would lead to the formation of pH sensor dye **3a** or carbocyanine dyes **4a** and **4b**, respectively. It is known that indolenine quaternary ammonium salts **I** and **IV** (Y=CMe_2) undergo elimination of acid halide even in neutral conditions to afford the isolable and stable Fischer's base **II** and **V** (Y=CMe_2), respectively,[16,17,21,22] whereas in the case of benzothiazole quaternary ammonium salt (Y=S) the acid halide elimination would be improbable because the methylenic base obtained necessarily requires basic conditions for the de-aromatization of the thiazole moiety. Therefore, it is expected that the yield and the reaction pathway of indolenine compared with benzothiazole one will be different. Indeed, indolenine led to the formation of pH sensor dye **3a** and indocarbocyanine dye **4a** in an overall 84% yield whereas benzothiazole led to the formation of only thiacarbocyanine dye **4b** in 8% yield.



Scheme 3. Proposed reaction mechanism for the synthesis of pH sensor dye **3a**.

Looking for further evidences for this proposed mechanism, we synthesized the heterocyclic salt and protonated forms similar to the intermediates postulated here, in order to verify whether their mixture in DMF would produce similar products to those indicated above. As shown in Scheme 4, the treatment of an ethereal solution of the heterocyclic base **1a**,**b** with HBr (48%) at room temperature affords the corresponding protonated form **IVa**,**b** quantitatively.[23]



Scheme 4. Synthesis of the pH sensor dye **5a**.

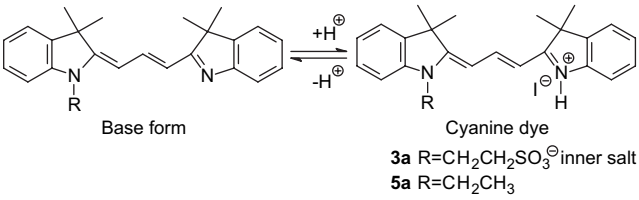
1-Ethyl-2,3,3-trimethylindolium (**I′a**) iodide and 1-ethyl-2-methylbenzothiazolium iodide (**I′b**) were prepared by refluxing a mixture of **1a** and **1b**, respectively, in the presence of an excess of ethyl iodide in acetonitrile.[24] DMF reflux of a mixture of **IVa** and **I′a** afforded the pH sensor dye **5a** together with its corresponding indocarbocyanine dye **6a**. Purification of the crude product by silica gel chromatography using gradients of MeOH/CH_2Cl_2 afforded the first fraction **5a** as a red crystal in 48% yield followed by the second one **6a** as a red crystal in 10% yield. Whereas in the case of **1b** the only obtained product was the thiacarbocyanine dye **6b** as a violet crystal in 8% yield after being purified in a similar manner to **6a**. These results would give not only an additional confirmation to the proposed mechanism but also did well reveal the differences between indolenine and benzothiazole behavior related to the reaction pathways.

Furthermore, DMF reflux for 20 h of one-pot reaction of ethyl iodide with indolenine in 1.14:2 molar ratio affords the same products **5a** and **6a** in 33 and 5% yield, respectively.

The structure of the new pH sensor dye **5a** was evidenced by its HRMS, ^{1}H NMR, ^{13}C NMR, and IR data. The ^{1}H and ^{13}C NMR spectral data for all dyes reported here were fully assigned with the aid of HMQC (Heteronuclear Multiple Quantum Coherence), HMBC (Heteronuclear Multiple Bond Correlation), and COSY (Correlated Spectroscopy) experiments.

2.3. pH-Dependent fluorescence and absorption spectra

As presented in Scheme 5, these pH sensors exist either as fluorescent cyanine dye or as complementary nonfluorescent base. The intense cyanine dye absorption and emission properties are mainly consequence of the resonance effect that exists due to the electronic push/pull effect between the two nitrogen atoms of the indole rings via the conjugated trimethine bridge. Therefore, abstraction of a proton from this system destroys this resonance, and subsequently leads to the nonfluorescent base form.



Scheme 5. Cyanine dye-base form of the pH sensors **3a** and **5a**.

The fluorescent properties of dyes **3a** and **5a** as a function of pH are shown in Figures 1 and 2. It can be observed that the fluorescent characteristics of the sensors are greatly reduced

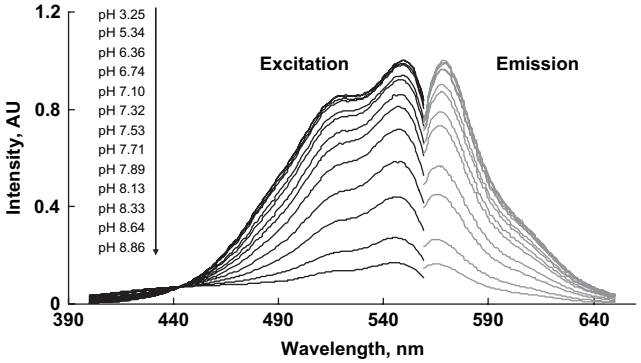


Figure 1. Excitation and emission spectra of dye **3a** (5×10^{-6} M, λ_{ex}= 550 nm and λ_{em}=569 nm) in aqueous buffer solutions at 25 °C as a function of pH.

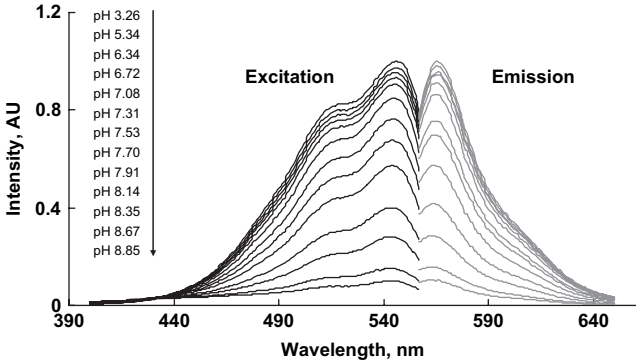


Figure 2. Excitation and emission spectra of dye **5a** (5×10^{-6} M, λ_{ex}= 546 nm and λ_{em}=565 nm) in aqueous buffer solutions at 25 °C as a function of pH.

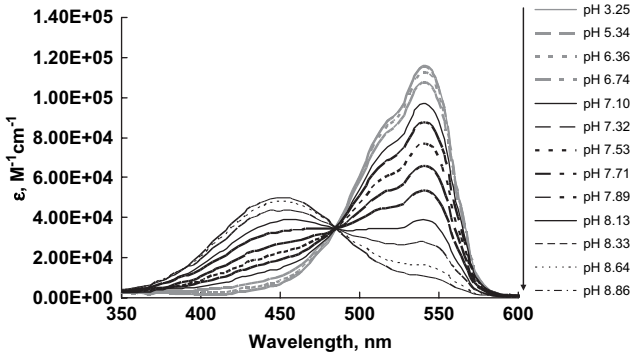


Figure 3. UV–visible absorption spectra of dye **3a** in aqueous buffer solutions (5×10^{-6} M) at 25 °C as a function of pH.

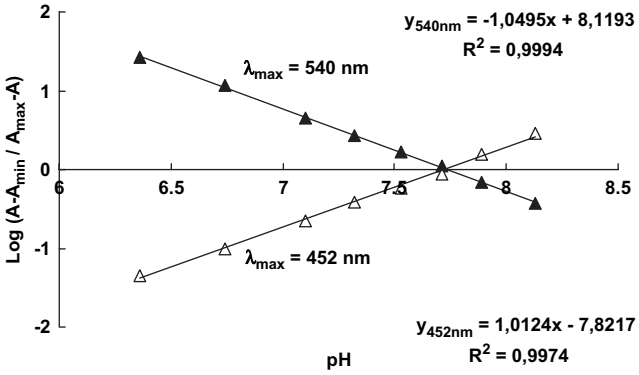


Figure 5. Determination of the pK_a value for dye **3a** (5×10^{-6} M) in aqueous buffer solutions.

as the buffer becomes less acidic, due to an increased deprotonation of the cyanine dye leading to a larger population of the nonfluorescent base species.

UV–visible spectra of both sensors as a function of pH prove the existence of acid/base equilibrium between the base and cyanine forms. It can be seen (Figs. 3 and 4) that as the buffer solutions become less acidic, the characteristic absorption maximum for the trimethine cyanine dye at 540 nm is greatly reduced as a new peak evolves at 452 nm. This absorption peak is due to the increased presence of the base form of the sensor by proton abstraction from the trimethine cyanine dye. Furthermore, the existence of an isosbestic point at 484 nm for both dyes reveals not only the presence of acid/base equilibrium between the two forms, but also gives a good indication that the solutions are all of similar ionic strength[25] and therefore minimizes the effect that others ions may cause on the spectral receptor in relation to protons. Figures 1 and 3 for sensor **3a** and Figures 2 and 4 for sensor **5a** show a coincidence between absorption and excitation indicating the purity of the sensors. Moreover, the existence of mirror image between excitation and emission spectra reveals a constant geometry of the molecule upon excitation.

The pH dependence of fluorescence intensity (F) shown in Figures 1 and 2 for dyes **3a** and **5a** can be analyzed using Eq. 1[26,27] in order to calculate their pK_a values. The pK_a may be defined as the pH, whereby 50% of the dye population in solution is protonated. The pK_a values can also be obtained from the pH dependence absorption intensity (A) shown in Figures 3 and 4 using Eq. 2,[28,29] where F_{max} or A_{max} is the maximum fluorescence or absorbance of the protonated form at a given wavelength and F_{min} or A_{min} is the minimum fluorescence or absorbance. pK_a of 7.73 and 7.53 were calculated for dyes **3a** and **5a**, respectively from the plot of log $(A-A_{min}/A_{max}-A)$ versus pH as shown in Figures. 5 and 6.

$$\mathrm{pH} = \mathrm{p}K_a \pm \log[(F_{max}-F)/(F-F_{min})] \quad (1)$$

$$\mathrm{pH} = \mathrm{p}K_a \pm \log[(A_{max}-A)/(A-A_{min})] \quad (2)$$

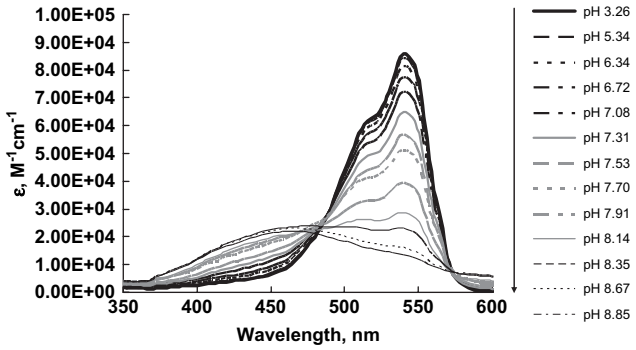


Figure 4. UV–visible absorption spectra of dye **5a** in aqueous buffer solutions (5×10^{-6} M) at 25 °C as a function of pH.

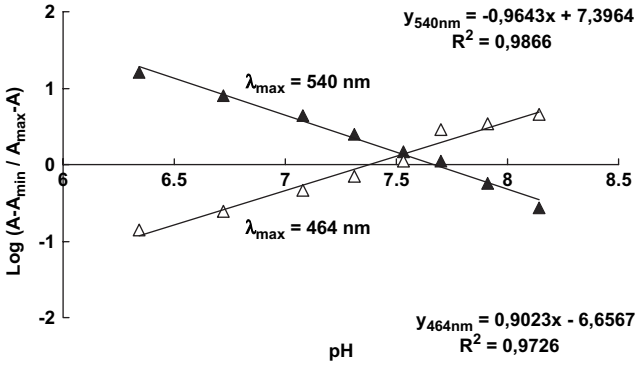


Figure 6. Determination of the pK_a value for dye **5a** (5×10^{-6} M) in aqueous buffer solutions.

3. Conclusions

A new Vilsmeier-type reaction is proposed for the synthesis of indocarbocyanine pH sensors. The in situ formation of Fischer's base reveals to be crucial for the preparation of the pH sensor dyes **3a** and **5a**, with a pK_a of 7.73 and 7.53, respectively. The observable changes of fluorescent emission within the critical intracellular pH range (6–8) make these probes very suitable for biological applications. The scope and limitation of the applicability of the presented reaction in the synthesis of different cyanine pH sensors will be the subject of a future work.

4. Experimental

4.1. General

All reagents were of the highest purity available, purchased from Sigma–Aldrich Company, and were used as received. Solvents were of analytical grade. Dry *N*,*N*-dimethylformamide and acetonitrile were used. All new dyes were determined to be >95% pure by ^{1}H NMR. All reactions were monitored by thin-layer chromatography (TLC) on aluminum plates precoated with Merck silica gel 60 F_{254} (0.25 mm) using dichloromethane or chloroform/methanol (5–30%) and the spots having been examined under 254, 312, and 365 nm UV light. Column chromatography was performed on silica gel 60 (70–230 mesh) from Macherey-Nagel, Germany. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 solution on a Brücker ACP 250 (250.13 and 62.90 MHz) or Brücker ARX 400 (400.13 and 100.62 MHz) spectrometers. Chemical shifts are reported in parts per million and coupling constants (*J*) are given in hertz. HMQC, HMBC, and COSY spectra were acquired on Brücker ARX 400 spectrometer. Infrared spectra were performed on a Mattson 5000-FTS FTIR spectrometer. All samples were prepared by mixing FTIR-grade KBr with 1% (w/w) compound and grinding to a fine powder. Spectra were recorded over the 400–4000 cm^{-1} range without baseline corrections. More intensive bands are given in inverse centimeter. High Resolution Fast Atom Bombardment Mass Spectra (HR FABMS) were recorded in a Micromass AutoSpec M, operating at 70 eV, using a matrix of 3-nitrobenzyl alcohol (3-NBA). Melting points were determined in open capillary tubes in a Büchi 530 melting point apparatus and are uncorrected. All pH values were determined by a 744 pH meter (Metrohm Instrument).

4.2. pH-Spectral changes

Stock solutions of the analyzed dyes (5×10^{-5} M) were prepared in ethanol from which 5 ml was mixed with 17.5 ml of McIlvaine buffer solution (pH 3.25–8.68)[30] containing 5 ml of 0.5 M sodium chloride to adjust the ionic strength. Then the mixture was made up to 50 ml by adding distilled water to obtain dye solutions in ethanol/water (1:9) mixture with concentration of 5×10^{-6} M. Absorption titration curves were made on an Unicam HeλIOSα spectrophotometer and the uncorrected fluorescence ones were made on a SPEX FluoroMax 3109 spectrofluorophotometer with the excitation and emission wavelengths indicated.

4.3. Synthesis

4.3.1. Typical procedure for the one-pot Vilsmeier-type reaction. A mixture of 2,3,3-trimethylindolenine **1a** (0.159 g, 1.0 mmol) and 2-bromoethanosulfonic acid sodium salt (0.317 g, 1.5 mmol) in DMF (2.0 ml) was refluxed for 48 h. After cooling to room temperature the reaction mixture was treated several times with ether to eliminate DMF and other impurities. The colored residue so formed was chromatographed on a silica gel column with gradients of MeOH/$CHCl_3$ (5–30%) to afford the first fraction containing the pH sensor dye **3a** as a dark red crystal in 32% and a second fraction as the corresponding symmetric indocarbocyanine dye **4a** as a dark red powder in 52% yield. Using the same procedure and starting from 2-methylbenzothiazole **1b** (0.149 g, 1.0 mmol), the unique dye obtained was the symmetric thiacarbocyanine dye **4b** as a violet powder in 8% yield.

4.3.1.1. 2-[3-(3,3-Dimethyl-1,3-dihydroindol-2-ylidene)-propenyl]-3,3-dimethyl-1-(2-sulfoethyl)-3*H*-indolium, inner salt (3a). Mp 229–232 °C, from $CHCl_3$/ether. IR (KBr) ν (cm^{-1}): 3445, 2924, 1564, 1474, 1466, 1395, 1175, 1148, 1109, 1034, 926, 748. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ (ppm): 1.47 (6H, s, 3″-(CH_3)$_2$), 1.67 (6H, s, 3-(CH_3)$_2$), 2.94 (2H, t, *J*=6.8 Hz, CH_2SO$_3$), 4.35 (2H, t, *J*=7.2 Hz, 1-NCH_2), 6.16 (1H, d, *J*=13.2 Hz, 3′-C*H*), 6.40 (1H, d, *J*=13.5 Hz, 1′-C*H*), 7.18 (1H, t, *J*=7.5 Hz, 5″-C*H*), 7.26 (1H, d, *J*=7.5 Hz, 7″-C*H*), 7.27 (1H, t, *J*=7.3 Hz, 5-C*H*), 7.36 (1H, t, *J*=7.4 Hz, 6″-C*H*), 7.38 (1H, d, *J*=7.1 Hz, 7-C*H*), 7.42 (1H, t, *J*=7.8 Hz, 6-C*H*), 7.53 (1H, d, *J*=7.3 Hz, 4″-C*H*), 7.61 (1H, d, *J*=7.4 Hz, 4-C*H*), 8.50 (1H, t, *J*=13.1 Hz, 2′-C*H*), 12.75 (1H, br s, 1″-N*H*). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ (ppm): 25.5 (3″-(*C*H$_3$)$_2$), 27.0 (3-(*C*H$_3$)$_2$), 41.1 (1-N*C*H$_2$), 47.4 (*C*H$_2$SO$_3$), 49.2 (3″-C), 49.3 (3-C), 100.8 (3′-*C*H), 102.0 (1′-*C*H), 111.3 (7-*C*H), 111.7 (7″-*C*H), 122.3 (4-*C*H), 122.8 (4″-*C*H), 124.0 (5″-*C*H), 124.9 (5-*C*H), 128.3 (6-*C*H, 6″-*C*H), 139.4 (3a″-*C*), 140.9 (3a-*C*), 141.3 (7a″-*C*), 141.8 (7a-*C*), 149.5 (2′-*C*H), 174.1 (2″-*C*), 177.0 (2-*C*). UV–visible (ethanol): λ_{max}=556 nm and ε_{max}=102,491 M^{-1} cm^{-1}. HRMS (FAB, 3-NBA): M+H, found 437.1900; $C_{25}H_{29}N_2O_3S$ requires 437.1899.

4.3.1.2. 3-(2-Sulfoethyl)-2-{3-[3-(2-sulfoethyl)-3*H*-benzothiazol-2-ylidene]-propenyl}-benzothiazol-3-ium (4b). Mp>300 °C; from water/acetone. IR (KBr) ν (cm^{-1}): 3399, 1557, 1447, 1425, 1343, 1209, 1036, 752. ^{1}H NMR (250.13 MHz, DMSO-d_6) δ (ppm): 2.99 (4H, t, *J*=7.0 Hz, CH_2SO$_3$), 4.51 (4H, br s, 1-NCH_2), 6.58 (2H, d, *J*=12.5 Hz, 1′-C*H*, 3′-C*H*), 7.35 (2H, t, *J*=7.6 Hz, 6-C*H*, 6″-C*H*), 7.52 (2H, t, *J*=7.4 Hz, 5-C*H*, 5″-C*H*), 7.63–7.73 (3H, m, 4-C*H*, 2′-C*H*, 4″-C*H*), 7.92 (2H, d, *J*=8.0 Hz, 7-C*H*, 7″-C*H*). ^{13}C NMR (62.90 MHz, DMSO-d_6) δ (ppm): 43.5 (*C*H$_2$SO$_3$), 47.9 (1-N*C*H$_2$), 99.3 (1′-*C*H, 3′-*C*H), 113.8 (4-*C*H, 4″-*C*H), 122.9 (7-*C*H, 7″-*C*H), 125.0 (7a-*C*, 7a″-*C*), 125.1 (6-*C*H, 6″-*C*H), 128.0 (5-*C*H, 5″-*C*H), 141.3 (3a-*C*, 3a″-*C*), 146.5 (2′-*C*H), 164.7 (2-*C*, 2″-*C*). UV–visible (water): λ_{max}=560 nm and ε_{max}=132,062 M^{-1} cm^{-1}. HRMS (FAB, 3-NBA): M$^+$, found 525.0264; $C_{21}H_{21}N_2O_6S_4$ requires 525.0282.

4.3.2. Typical procedure for the confirmation of the proposed mechanism. A mixture of the protonated indole form **IVa** (0.293 g, 1.22 mmol) and the salt **I′a** (0.281 g, 0.89 mmol) in DMF (4 ml) was refluxed for 20 h. After cooling to room temperature the reaction mixture was treated several times with 0.1 N HCl followed by distilled water to eliminate DMF and other impurities. The colored residue so formed was chromatographed on a silica gel column with gradients of MeOH/CH_2Cl_2 (5–20%) to afford the first fraction as the crude yellow dye **5a**, which was re-dissolved in dichloromethane and treated with 1 N HI solution (20 ml) and 14% KI (20 ml). The aqueous layer was further extracted with dichloromethane and the combined extracts were dried with anhydrous sodium sulfate and evaporated. Crystallization from chloroform/petroleum ether affords

dye **5a** as a dark red crystal in 48% yield. The second fraction so obtained was the corresponding symmetric indocarbocyanine dye **6a** as a dark red crystal in 10% yield, mp 273–276 °C (260–263 °C).[19a] Using the same procedure and starting from protonated benzothiazole **IVb** (0.234 g, 1.02 mmol) and salt **I′b** (0.315 g, 1.02 mmol), the unique dye so obtained was the symmetric thiacarbocyanine **6b** as a violet crystal in 8% yield, mp 267–268 °C (269 °C).[19a]

4.3.2.1. 2-[3-(3,3-Dimethyl-1,3-dihydroindol-2-ylidene)-propenyl]-1-ethyl-3,3-dimethyl-3*H*-indolium iodide (5a). Mp 140–142 °C; from $CHCl_3$/petroleum ether. IR (KBr) ν (cm^{-1}): 2967, 1562, 1491, 1464, 1379, 1194, 1115, 1079, 928, 752. ^1H NMR (250.13 MHz, DMSO-d_6) δ (ppm): 1.29 (3H, t, J=6.7 Hz, CH_2CH_3), 1.47 (6H, s, 3″-$(CH_3)_2$), 1.71 (6H, s, 3-$(CH_3)_2$), 4.17 (2H, q, J=7.4 Hz, 1-NCH_2), 6.19 (1H, d, J=13.0 Hz, 3′-C*H*), 6.44 (1H, d, J=13.9 Hz, 1′-C*H*), 7.20 (1H, t, J=7.4 Hz, 5″-C*H*), 7.28–7.46 (5H, m, 5-C*H*, 6-C*H*, 7-C*H*, 6″-C*H*, 7″-C*H*), 7.55 (1H, d, J=7.2 Hz, 4″-C*H*), 7.68 (1H, d, J=7.5 Hz, 4-C*H*), 8.50 (1H, t, J=13.2 Hz, 2′-C*H*), 12.76 (1H, br s, 1″-N*H*). ^{13}C NMR (62.90 MHz, DMSO-d_6) δ (ppm): 12.3 (CH_2CH_3), 25.8 (3″-$(CH_3)_2$), 27.3 (3-$(CH_3)_2$), 38.5 (1-NCH_2), 49.2 (3″-C), 49.4 (3-C), 100.5 (3′-*C*H), 101.9 (1′-*C*H), 111.3 (7-*C*H), 111.8 (7″-*C*H), 122.6 (4-*C*H), 123.1 (4″-*C*H), 124.1 (5″-*C*H), 125.2 (5-*C*H), 128.5 (6-*C*H, 6″-*C*H), 139.3 (3a″-*C*), 141.4 (3a-*C*, 7a-*C*, 7a″-*C*), 149.7 (2′-*C*H), 173.9 (2″-*C*), 176.9 (2-*C*). UV–visible (ethanol): λ_{max}=552 nm and ε_{max}=82,511 $M^{-1}\,cm^{-1}$. HRMS (FAB, 3-NBA): M^+, found 357.2325; $C_{25}H_{29}N_2$ requires 357.2330.

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