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pH-Dependent Optical Properties of Synthetic Fluorescent Imidazoles

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Abstract

An imidazole moiety is often found as an integral part of fluorophores in a variety of fluorescent proteins and many such proteins possess pH dependent light emission. In contrast, synthetic fluorescent compounds with incorporated imidazoles are rare and have not been studied as pH probes. In this report, the richness of imidazole optical properties, including pH sensitivity, was demonstrated via a novel imidazole-based fluorophore 1H-imidazol-5-yl-vinyl-benz[e]indolium.

Three species corresponding to protonated, neutral and deprotonated imidazoles were identified in the broad range of pH 1-12. The absorption and emission bands of each species were assigned by comparative spectral analysis with synthesized mono- and di-N-methylated fluorescent imidazole analogues. pKa analysis in the ground and the excited states showed photoacidic properties of the fluorescent imidazoles due to the excited state proton transfer (ESPT). This effect was negligible for substituted imidazoles. The assessment of a pH sensitive center in the imidazole ring revealed the switching of the pH sensitive centers from 1-N in the ground state to 3-N in the excited state. The effect was attributed to the unique kind of the excited state charge transfer (ESCT) resulting in a positive charge swapping between two nitrogens.

Keywords

emission spectroscopy; imidazole; pH sensitivity; energy transfer; charge transfer

Introduction

Fluorescent molecular pH sensors are widely used to control and monitor chemical reactions and biological processes. A majority of the available synthetic fluorescent probes employ tunable acidity of phenyls (naphthols, fluoresceins and pyranines) and cyclic aromatic amines (oxazoline, cyanines, and porphyrines)[1-5] to create pH sensitive centers. Interestingly, fluorescent proteins with a seemingly limited choice of available pH sensitive groups utilize mostly the same set of functionalities: phenyls,[6] aromatic amines,[7] and oxazoles.[8]

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Fluorescent imidazoles show rich pH dependent optical properties

Fluorescent imidazoles: Peek into the structure of fluorescent proteins led to the synthesis of fluorescent imidazoles. Prepared compounds demonstrated an array of remarkable pH dependent optical properties including and at least two types of excited state charge transfers.

However, in addition to common groups, some fluorescent proteins also employ imidazole as a pH sensitive functionality (class 6 according to Tsien classification.[9]) For example, imidazole is a key constituent of the fluorophore system of red fluorescent proteins EBFP2, mCherry, and EosFP,[7,10] (Figure 1) and apparently is responsible for their pH sensitivity. [11-13] To the best of our knowledge, there have been no reports on utilizing imidazole moieties in synthetic pH probes despite the attractive optical properties of imidazole containing fluorophores, such as high quantum yield and pH dependence.

The long standing interest of our lab lies in synthesis of novel near-infrared probes and their applications in biological imaging. Recently, we initiated a screening of organic functionalities which could be suitable as pH sensors and synthetically appropriate for incorporation into a polymethine skeleton. Here, inspired by the optical properties of fluorescent proteins, we report our first experimental data where the new molecular construct was based on incorporating imidazole heterocycle via a methine linker into a fluorogenic π -conjugated benz[e]indolium core (compound **2** Scheme 1). Optical study of the synthesized molecules revealed a number of interesting and sometimes unexpected observations. We observed three modes of pH sensitivity in the range of pH 1-12 attributed to the protonated, neutral and deprotonated forms of imidazole with different degree of fluorescence. There existed at least two channels of the excited state charge transfer (ESCT) at certain pH: within the imidazole ring and between the imidazole ring and benz[e]indolium. The interpretation of pH sensitivities in both ground and excited states and the detailed investigation of the related underlying mechanisms were the goals of this study.

Results and Discussion

1. Synthesis of fluorescent imidazoles

To understand the mechanism of pH sensitivity, we prepared and examined optical properties of imidazole **2** and compared them with related compounds such as di-N-methylated imidazolium **3** and mono-N-methylated imidazoles **4** and **5**, an approach often used in the literature to explore the mechanism of protonation.[14-16] Fluorescent imidazoles **2-4** were prepared via electrophilic substitution by mixing imidazole-bearing aldehydes prepared in our lab[17] or from commercial sources with a known fluorophore building block 3-(2-carboxyethyl)-1,1,2-trimethyl-1H-benz[e]indolium **1** (Scheme 1). The pendant carboxylate group in **1** was selected for potential further conjugation with bioactive molecules.[4] With stoichiometric ratio of the starting materials, the conversion was nearly quantitative. Compounds **2**, **4** and **5** were made as internal salts from the pairing of a carboxylate ion with the positively charged benzoindolium; compound **3** is a BF_4^+ salt.[17]

While imidazoles **2**, **3** and **4** could be easily made from commercially or readily available imidazole carbaldehydes,[17] the preparation of 3-N-methyl imidazole **5** presented a certain challenge due to the lack of regioselective imidazole methylation on 3-N position. Our initial efforts based on proposed literature methods led to a mixture of isomers[18] or required protection-deprotection schemes,[19,20] which resulted in lower yield. Finally, we chose methylation of 5-imidazolecarbaldehyde with a stoichiometric amount of methyl iodide. The mixture of 1-N methyl and 3-N methyl derivatives (ratio 2:3, ^1H NMR) was found to be difficult to separate. After the aqueous work-up, the mixture was allowed to react with benz[e]indolium **1** via electrophilic substitution in the next step. The reaction mixture containing two products with the same molecular masses was easily separated into two constituent compounds via reverse phase chromatography. The ^1H NMR and LCMS spectra of the first, more hydrophilic elutant were identical to the compound alternatively prepared from commercially available 1-N-methyl imidazole carbaldehyde. The second, more hydrophobic product, is most likely the 3-N-methyl imidazole **5** isomer.

To confirm their identity, compounds **4** and **5** were fully characterized by ^1H and ^{13}C NMR, including COSY, HMQC and HMBC. The final assignment of an N-methyl group position was made by ^1H NOE experiments (Figure 2 and SI). Irradiation of compound **4** with frequency $\delta = 4.17$ ppm (H-16) led to the observed signal enhancements of the two nearest protons, H-12 (8.18 ppm) and H-15 (8.92 ppm), confirming that the methyl group was attached to the 1-N position. In contrast, irradiation at 3.89 ppm (H-16) of the second elutant resulted in the enhancement of H-14 (8.05 ppm) and H-15 (8.22 ppm), suggesting that the methyl group is connected to the 3-N position and confirming that the second elutant is 3-N-methyl substituted regioisomer **5**. In addition, strong NOE between H-12 and H-9 (not shown) and the absence of NOE between H-14 and H-12 (crossed dotted arrow) indicated *trans-cis* conformation of compound **4**. In contrast, strong NOE between H-12 and H-9 (not shown) and strong NOE between H-14 and H-12 (dotted arrow) indicated *trans-trans* conformation of **5**. [21]

2. Absorption and emission spectra assignment

For the analysis of pH-dependent properties, the spectra were recorded in water between pH 1.0 and 12.0 using diluted HCl or NaOH for pH adjustment. At pH ~ 6 , compound **2** showed a broad absorption band at 430 nm and a smaller band at 360 nm (Figure 3, left). At lower pH, the absorption of the visible band became smaller with simultaneous increase of the near-UV band with a diffuse isosbestic point at 370 nm. Below pH ~ 2.9 , the 360 nm band became dominant and no further changes in absorption band were observed past pH ~ 2.5 . Under basic conditions, the absorption band experienced a red shift to 477 nm with a well defined isosbestic point at 440 nm (Figure 3, right). The presence of two independent isosbestic points clearly suggested the existence of two titration centers and therefore the presence of the three states, which we tentatively assigned as the protonated, neutral and deprotonated forms of imidazole (Scheme 2).

In an acidic environment, compound **2** revealed a broad and almost structureless fluorescence at 600 nm (Figure 4, left). Under neutral conditions, the fluorescence became much weaker and blue shifted to 560 nm with an isoemissive point at 525 nm. Further basification increased emission intensity upon excitation at 460 nm, accompanied by a pronounced emission shift to 530 nm (Figure 4, right). The change in absorbance and emission titration curves as a function of pH revealed three well separated regions in both ground and the excited states (Figure 5). Optical properties of the compounds under acidic, neutral and basic conditions are given in Table 1.

All species showed similar molar absorptivity values at given pH and exhibited a general upward trend with pH increase (from 9,000- 16,000 $\text{M}^{-1} \text{cm}^{-1}$ in acidic solutions to 21,000 – 25500 $\text{M}^{-1} \text{cm}^{-1}$ in basic media, Table 1). Similarly, large and pH dependent Stokes shifts ($\sim 11,000 \text{ cm}^{-1}$ at pH 2, $\sim 5,000 \text{ cm}^{-1}$ at pH 7, and $\sim 2,000 \text{ cm}^{-1}$ at pH 11). Quantum yields and fluorescence lifetimes for studied molecules were rather low in water but significantly increased in a constrained environment, such as highly viscous media, which typically exist inside proteins. Indeed, relatively weak fluorescence of **2** in aqueous solutions with fluorescence quantum yield $\Phi = 0.014$ and lifetime $\tau = 0.5 \text{ ns}$ increased significantly to $\Phi = 0.15$, $\tau = 1.19 - 1.28 \text{ ns}$ in glycerol.

Acidic and neutral media—To assign absorption and emission bands, we first considered acidic and neutral conditions. Compound **3**, previously synthesized in our laboratory [10] and possessing a permanent cationic charge set due to the exhaustive N-methylation of imidazole, was used as an isoelectronic analogue of the protonated imidazolium 2H^+ . As expected, **3** was not pH sensitive (no spectral shift in acidic and neutral pH, Figure 6), which correlated well with the lack of “active solvation centers” [22] in the molecule. The change in molar absorptivity and quantum yield of **3** from neutral to acidic pH was much smaller than the

corresponding changes in compound **2** (Table 1). The absorption and the emission spectra of the compound **2H⁺** in acidic media and **3** in both acidic and neutral solutions clearly resembled each other, suggesting electronic similarity between the two entities. Fluorescence lifetime measurements of **2H⁺** and **3** showed that both molecules had the same lifetime of 0.5 ns. The similarities of the spectral parameters (position, shape and lifetime) of **2H⁺** and **3** suggested that the absorption at 360 nm and emission at 600 nm band belonged to the protonated imidazolium **2H⁺** (Scheme 2). In the fluorescence spectra of **3**, the absence of the band at 560 nm spectra seen for **2** and **2H⁺** under the neutral and basic conditions correlated well with the absence of the neutral form of **3**. Thus, the emission at 560 nm in neutral pH of **2** was unequivocally assigned to the neutral imidazole (Scheme 2).

Basic media—Having assigned protonated and neutral forms, attention was given to compound **2** under basic conditions (**2H⁻**). At pH greater than 9, the absorption spectra showed a dramatic change characterized by a red shift to 477 nm and significant increase in absorption intensity (Figure 3, right). Simultaneously, fluorescence experienced a substantial blue shift to 530 nm (Figure 4, right). These changes were unique to **2** among the four studied imidazoles and were attributed to the formation of imidazole anion **2H⁻_a** or its resonance form **2H⁻_b** via the deprotonation of the imidazole ring (Scheme 2). Unlike **2**, the absorption spectra of compounds lacking labile N-H protons such as **4** and **5** under basic conditions showed no bathochromic shifts and no new isosbestic points (Figure 8), suggesting the absence of respective deprotonated forms. Indeed, the absorption spectra of **4** and **5** under basic conditions were similar to the absorption spectra of the neutral imidazole **2** (Figure 3 and 8). Also the absence of blue shifted fluorescent peaks under basic conditions for **4** and **5** at 530 nm (Figure 9) further supported the absence of deprotonation for these molecules. Thus, 477 band nm in absorption spectra of **2** was assigned to their deprotonated forms **2H⁻_{a,b}**.

The pK_a ~ 10.7 of the **2**↔**2H⁻** deprotonation in both the ground state and the excited state was determined. Such pK_a is unusually low for imidazoles since the deprotonation of imidazoles to their anionic form, imidazoles, is typically higher with pK_a=14.5[23] and necessitates very strong bases. However, in the presence of certain functionalities, the pK_a can be lowered significantly. For example, the presence of electron withdrawing groups might shift the dissociation constant to lower pK_a values; i.e. for urocanic acid, pK_a=13[20] has been observed. Furthermore, conjugation of the imidazolate ring to a positively charged group (e.g. benz[e]indolium) can lower the pK_a even more due to the energetically favourable loss of overall charge and the formation of a neutral conjugated chromophore **2H⁻_b** (a related case was recently described by Oto et al.[24])

Among neutral, protonated, and deprotonated forms of **2**, the protonated form demonstrated the highest fluorescence (Table 1). The decrease of fluorescence and the blue shift from 600 nm to 530-560 nm (Figure 4) was apparently caused by photo-induced electron transfer originating from an unshared electron pair located in imidazole nitrogens[25] that is electronically coupled with a chromophore. Under acidic conditions, the electron pair was deactivated via protonation and did not compete with the radiative decay on the same level. For that reason, the emissions of the electronically identical **2H⁺**, di-N-methylated **3**, and mono-N-methylated protonated **4H⁺** and **5H⁺** were found to be quite similar (Figure 7); in all the structures the electron pair was blocked.

3. Determination of the pH sensitivity nitrogen center – pK_a analysis

Having established the correlation between imidazole structures and their optical properties, we closely examined the behavior of the protonated molecules to establish which nitrogen in the imidazole ring of **2** is responsible for pH sensitivity. Careful analysis of Figure 7 revealed that, despite an obvious similarity in emission profiles between the studied imidazoliums, the

emission profile of 2H^+ fully overlapped only with the emission of 5H^+ , suggesting significant resemblance between these two excited species. In addition, detailed pKa analysis showed that the sensitivities in the ground state and in the excited state were different: 1-N was the pH sensitive centre in the ground state and 3-N in the excited state.

The pKa of the studied compounds in the ground and excited states were determined from the corresponding absorption and emission spectra (Figure 3, 4, 8 and 9) using principal component analysis (PCA) and validated with sigmoidal dose-response curve fits (see SI) from titration diagrams. The results are tabulated in Table 2.

Imidazole 2H^+ exhibited photoacidic properties, known as excited state proton transfer (ESPT).[26-29] In the excited state, the pKa was 1.4 pH units lower than the pKa of the ground state. Calculated molar ratios for each of the principal components (from PCA) as a function of pH are shown in Figure 10. Each of the three panels represents a photolytic equilibrium of two principal components identified in the ground and the excited state. The point of the intersection of both components at each panel corresponds to the pKa values. The intersections of absorption curves correspond to the pKa in the ground state, while intersections of the fluorescence curves correspond to the pKa in the excited state. The top panel describes the equilibrium between the neutral imidazole **2** and its protonated form 2H^+ ($2 \leftrightarrow 2\text{H}^+$). Clearly, the intersections of absorptions and fluorescence differed. The pKa found for the excited state ($\text{pKa}^* = 2.82$) was markedly lower than that in the ground state ($\text{pKa} = 4.20$), indicating photoacidic properties. The same approach for imidazole 4H^+ revealed substantially less photoacidity than 2H^+ and negligible photoacidity for compound 5H^+ .

The results shown in Figure 10 clearly illustrate which nitrogen in the imidazole ring is responsible for pH sensitivity in the ground and excited states. The dissociation constants of 2H^+ in the ground state were identical to the dissociation constant of 4H^+ (green line) in the ground state, suggesting similar mechanisms of protonation/deprotonation. Indeed, it is generally accepted that the 3-N position in imidazoles is preferably protonated under acidic conditions due to the lone electron pair localized on the 3-N position[30]. The unshared electron pairs in **2** and **4**, which are localized on the 3-N position, are equally available for protonation and thus apparently have similar basicity as indicated by their identical pKa values, which suggests the isoelectronic configuration of 2H^+ and 4H^+ in the ground state. In contrast to the ground state, the excited state of imidazole 4H^+ showed substantially higher basicity ($\text{pKa}^* \sim 3.87$) than 2H^+ in its excited state ($\text{pKa}^* \sim 2.82$), demonstrating the divergence of their electronic configurations in the excited state. In contrast, the dissociation constants in the excited states of 2H^+ and 3-N-Me 5H^+ ($\text{pKa}^* \sim 2.85$) were nearly identical, suggesting *isoelectronic configurations of 2H^+ and 5H^+* in their excited states. Thus, imidazole 2H^+ is similar to 4H^+ in the ground state and 5H^+ in the excited state.

To explain this phenomenon, we propose a mechanism based on excited state charge transfer, where the rearrangement in electron density upon excitation leads to a swap of a positive charge between 1-N to 3-N. Upon excitation, the electron density in the imidazole ring rearranges in such a way so that the positive charge moves from the 3-N position in the ground state to the 1-N position in the excited state, as shown in a modified Förster cycle (Scheme 3). In this scheme, the ground state 2H^+ resembles the ground state of 4H^+ (the resemblance is shown by a blue double-headed arrow) and the excited state 2H^+ resembles 5H^+ (red double-headed arrow). Upon excitation, the redistribution of charges in 2H^+ takes place, resulting in a shift of electron density from one nitrogen to another.

Conclusions

An imidazole incorporated into a fluorophore showed a wealth of pH dependent optical properties. Investigation into the mechanisms of pH sensitivity was accomplished by synthesizing the mono- and di-N-methylated analogues and provided the following findings: *i.* Fluorescent imidazole was found to be pH sensitive in all three ranges of the pH scale from 2.0 to 11.0. Three distinct absorption bands at 360, 430 and 477 nm in acidic, neutral and basic conditions were assigned to the protonated, neutral and deprotonated forms of imidazole. The deprotonated and neutral forms of fluorescent imidazole emitted at 530 and 560 nm correspondingly; in acidic condition, the emission shifted to 600 nm. *ii.* 1-N was determined to be a pH sensitive centre in the ground state while 3-N was responsible for pH sensitivity in the excited state. Such switching between the centers was attributed to the excited state charge transfer (ESCT) from one nitrogen to another, resulting in a positive charge swapping between two nitrogens. *iii.* pKa analysis in the ground and the excited state showed photoacidic properties of the studied fluorescent imidazole due to excited state proton transfer (ESPT); this effect was found negligible for substituted imidazoles and under basic conditions. *iv.* Large Stokes shifts of the dyes in protonated form and much shorter Stokes shift in the deprotonated form clearly indicate the presence of pH dependent twisted intramolecular charge transfer (TICT). The mechanism of this channel as well as theoretical experimental data confirming the presence of the process will be the focus of further study.

Experimental Section

General procedure for conjugating imidazolecarbaldehydes to benzo[e]indolium

To the of imidazole carbaldehyde (1.0-1.1 eq.) in methanol, 3-(2-carboxyethyl)-1,1,2-trimethyl-1H-benzo[e]indolium 1[31] bromide (1.0 eq.) was added, followed by the addition of sodium acetate (1.5 eq.) The reaction was stirred for 20 h at room temperature in the dark. The final product was isolated using reverse phase, medium pressure flash chromatography (Biotage AB, Sweden) with gradient 10% - 95% acetonitrile in water (both solvents contained TFA (0.1%)). NMR spectra were recorded at 300 MHz GE Omega and at 600 MHz Varian NMR spectrometers. LC/MS-ESI analysis in positive mode was conducted on Shimadzu LCMS 2010A equipped with UV-Vis and fluorometer detector using reverse phase C-18 column.

Optical measurements

UV/Vis spectra of samples predissolved in methanol and diluted with water were recorded using Beckman-Coulter DU-640 spectrophotometer. Fluorescence spectra were recorded using Fluorolog III (Horiba Jobin Yvon Inc., Edison NJ) fluorometer using excitation at 400 nm unless indicated. Quantum yields of species at acidic and neutral pH (excitation 400 nm) were recorded relatively to quinine hemisulfate monohydrate in 0.1M H₂SO₄ in water ($\Phi=0.54$ [32]); for basic species (excitation 460 nm) quantum yield was recorded relatively to fluorescein in 0.1N NaOH in water ($\Phi=0.95$. [32]) Fluorescence lifetime was measured using time correlated single photon counting (TCSPC) technique (Horiba) with excitation sources NanoLed® 370 and 460 nm and R928P detector (Hamamatsu Photonics, Japan) set to 600 nm or 560 nm respectively. The instrument response function was obtained using Rayleigh scatter of Ludox – 40. DAS6 v6.1 decay analysis software (Horiba) was used for lifetime calculations.

Titration experiments

Compounds **2-5** were dissolved in methanol (0.2 mL) and added into a beaker with water (100 mL) under stirring. Flow of argon was constantly delivered to the top of the solution to keep CO₂ out. The solution was basified with diluted aqueous NaOH and the desired pH was attained by titrating with aqueous HCl, or backwards at relatively low ionic strengths ($I = 0.02-0.05$

M). The pH of the solution was continuously measured using Accumet pH meter AB15 (Fisher Sci.) pKa values were calculated using principal component analysis software (DATAN 3.1, MultiD Analyses AB, Sweden) and sigmoidal dose-response curve fit implemented in software Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

For optical measurements in glycerol, compound **2** was pre-dissolved in methanol, and 10uL the methanolic solution (10 uL) was added to cuvettes with neutral and acidic glycerol (1 mL) (acidified with TFA, total volume of TFA 0.06 vol%). The glycerol solutions were stirred with a small spatula directly in a cuvette and allowed to stand for 2 hours in dark for complete homogenation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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21. In compound **4**, fast H/D exchange in methanol-*d*₄ precluded the observation of NOE between H-11 and H-14, which would be a direct proof of *trans-cis* conformation in **4**. In compound **5** the H/D

exchange was insignificant and the absence of NOE between H-11 and H-14 confirmed the *trans-trans* conformation of **5**.

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Scheme 1.
Synthesis of imidazole (Im) containing fluorophores.

Scheme 2.

Suggested transformations of **2** under acidic, neutral and basic conditions. pK_a and pK_a^* were determined for the ground and excited states, respectively.

Scheme 3.

Proposed Förster cycle for imidazole compounds, illustrating the similarity between $2H^+$ and $4H^+$ in the ground state and between $2H^+$ and $5H^+$ in the excited state.

Figure 1. Fluorophores from red emitting proteins: mCherry,[7] EBFP2,[7] and EosFP.[10]

Figure 2. Interactions between protons used to assign the position of the methyl group by NOE. ^1H NOE enhancement: **4** (left) – $\delta = 4.17$ ppm to 8.18 and 8.92 ppm: H-16 to H-12 and H-15; **5** (right) – $\delta = 3.89$ ppm to 8.05 and 8.22 ppm: H-16 to H-14 and H-15.

Figure 3. Absorption spectra of **2** under acidic (left) and basic (right) conditions in water. Absorbance increases with the increase of pH and shifts to longer wavelengths.

Figure 4. Fluorescence spectra of compound **2** at pH from 1.7 to 6.4 (left, excitation 400 nm) and at pH 7.0-11.51 (right, excitation 460 nm).

Figure 5. Titration absorption spectra of compound **2** (left) and titration emission spectra (right, excitation and emission as indicated).

Figure 6.

Absorption and emission spectra of **3** under acidic and neutral conditions in water (excitation 400 nm). The overall change in quantum yield for compound **3** was less than 30%.

Figure 7. Normalized fluorescence spectra of compounds **2-5** at pH 2. Excitation 400 nm. Emission traces of **2H⁺** and **5H⁺** completely overlap. Emission traces of **3** and **4H⁺** are shifted 7-8 nm.

Figure 8.

Absorption spectra of **4** (left) and **5** (right) at pH 1.0 - 11.5. Absorbance increases with increasing pH. The existence of only one isosbestic point suggests the presence of only two species: protonated and neutral forms.

Figure 9.

Fluorescence spectra of compounds **4** (left) and **5** (right), excitation 400 nm. Fluorescence decreases with increasing pH and remains stable after pH 6. No emission signals at 530-560 nm at basic pH suggests the absence of deprotonated forms.

Figure 10. Calculated molar ratio for each of the components as a function of pH using PCA. The intersection of the two molar ratio curves corresponds to the pKa of the protolytic equilibrium.

Table 1

Molar absorptivities and quantum yields of compounds **2-5** in water at different pH.

Entry	pH 2		pH 7		pH 11	
	ϵ , M ⁻¹ cm ⁻¹	ϕ^a ,	ϵ , M ⁻¹ cm ⁻¹	ϕ^d ,	ϵ , M ⁻¹ cm ⁻¹	ϕ^f ,
2	9200	0.014	15400	0.002	25500	0.004
3	16000	0.006	15500 ^c	0.008 ^c	degradation	
4	11400	0.008	15300	0.001		
5	12100	0.013	15100	0.002	21000	n/f
					21100	n/f

ϵ – molar absorptivity obtained at max absorption wavelength

n/f – non-fluorescent

^a excitation 400 nm

^b excitation 460 nm

^c pH 6.2, at higher pH, significant decomposition has been observed

^d quantum yield ϕ relative to quinine in 0.1M H₂SO₄

^f quantum yield ϕ relative to fluorescein in 0.1N NaOH

Table 2

pKa of fluorescent imidazoles in acidic media in the ground and excited states, calculated from PCA and titration diagrams (in parenthesis)

	$2 \leftrightarrow 2\text{H}^+$	$4 \leftrightarrow 4\text{H}^+$	$5 \leftrightarrow 5\text{H}^+$
Ground state, pKa	4.20 (4.20±0.05)	4.22(4.39±0.03)	2.68 (2.75±0.08)
Excited state, pKa*	2.82 (3.00±0.03)	3.89 (4.07±0.05)	2.85 (2.72±0.06)