ORGANIC LETTERS

2011
Vol. 13, No. 17
4510–4513

A New Half-Condensed Schiff Base Compound: Highly Selective and Sensitive pH-Responsive Fluorescent Sensor

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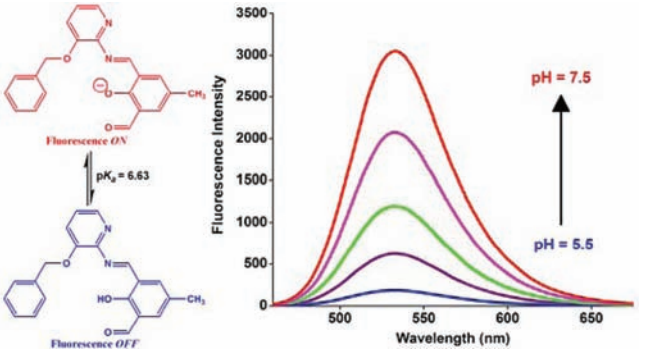
Received June 20, 2011

ABSTRACT



A new probe, 3-[(3-benzyloxypyridin-2-ylimino)methyl]-2-hydroxy-5-methylbenzaldehyde (1-H) behaves as a highly selective fluorescent pH sensor in a Britton–Robinson buffer at 25 °C. The pH titrations show a 250-fold increase in fluorescence intensity within the pH range of 4.2 to 8.3 with a pK_a value of 6.63 which is valuable for studying many of the biological organelles.

Fluorescent probes are powerful tools in cell biology for the nondestructive measurement of intracellular species to clarify the real-time dynamics and various biological functions of targeted metal cations in living cells owing to their simplicity and sensitivity.[1] Biochemical processes frequently involve protonation and deprotonation of biomolecules with concomitant changes in the pH of the environment in many cellular events, such as cell growth,[2] calcium regulation,[3] endocytosis,[4] chemotaxis,[5] and other cellular processes.

As minor variations of intracellular pH may induce cellular dysfunction, development of a desirable highly

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10.1021/ol201652r
Published on Web 08/09/2011

sensitive pH fluorescent probe having excitation profiles in the visible region is a cynosure for chemists. A limited number of pH-responsing fluorescent probes have been developed to monitor diverse physiological and pathological processes.[6] Limitations of the currently available pH probes include low sensitivity and/or excitation profiles in the ultraviolet region. Among the fluorescent pH probes that have been reported, only a small number are practical for intracellular imaging.[7,8]

To overcome these problems, we have designed and synthesized a new probe, a simple half-condensed Schiff-base compound 3-[(3-benzyloxypyridin-2-ylimino)methyl]-2-hydroxy-5-methylbenzaldehyde) (**1-H**) which behaves as a highly selective fluorescent pH receptor in a Britton–Robinson buffer at 25 °C and it shows a 250-fold enhanced fluorescence when pH is shifted from 4.2 to 8.3. Interestingly, the presence of an excess of the biologically relevant (Na^+, K^+, Ca^{2+}, etc.) and other metal (Cr^{3+}, Mn^{2+}, Fe^{3+}, etc.) ions does not affect the intensity. To the best of our knowledge, this type of simple half-condensed Schiff-base type compound as fluorescent probe for sensing pH for cellular imaging in living cells is still unexplored.

The synthesis of **1-H** involves the addition of a solution of 2,6-diformyl-4-methylphenol in methanol to a stirred methanolic solution of 3-benzyloxypyridin-2-ylamine in a 1:1 ratio. Single crystals of **1-H** for X-ray analysis were obtained from the solution of **1-H** in acetonitrile on slow evaporation. The molecular view of the title compound **1-H** with atom labeling scheme is shown in Figure 1, and the crystal data and relevant refinement parameters are tabulated in Table S1 (Supporting Information). The crystal packing in **1-H** is influenced by C–H---O hydrogen bonds (C13---O2 3.297(6) Å; H13---O2 2.37 Å; C13–H13---O2 172°) and π---π interactions as shown in Figure S2 (Supporting Information).

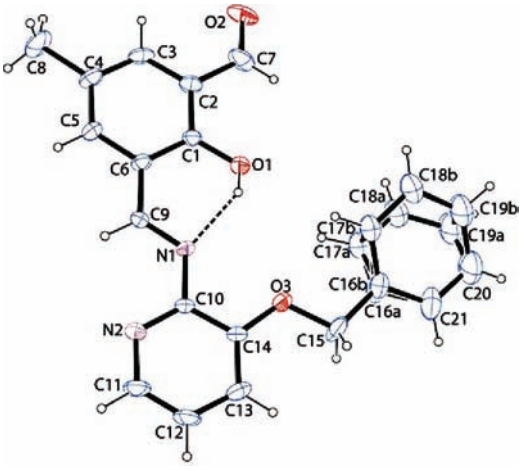

Figure 1. ORTEP view of $C_{21}H_{18}N_2O_3$ (**1-H**) with atom numbering scheme. The terminal phenyl ring is disordered over two positions [(C16A–C19A–C20–C21) and (C16B–C19B–C20–C21)] with a site occupancy ratio of 0.54(2):0.46(2). The intramolecular O–H---N hydrogen bond is shown by the dotted line.

The fluorescence spectrum of **1-H** in Britton–Robinson buffer excited at 440 nm exhibits a fluorescence maximum (Figure S3, see the Supporting Information) at 528 nm at 25 °C in the low pH range. With an increase in pH, the fluorescence intensities rapidly increase with a concomitant red shift, and finally the fluorescence maximum at 533 nm was observed. Fluorescence intensities of the probe (**1-H**) were measured with the gradual increase of pH from 4.00 to 9.00 (Figure 2). This fluorescence increase with increase in pH is accounted by the formation of **1** (phenoxide)[9] in higher pH (Scheme 1). The analysis of fluorescence intensity changes as a function of pH by using the Henderson–Hasselbalch[10] equation: $-\log[(FI_{max} - FI)/(FI - FI_{min}) = pH - pK_a$ where FI is the observed fluorescence intensity at a fixed wavelength, FI_{max} and FI_{min} are the corresponding maximum and minimum respectively, yielded a pK_a of 6.63 (±0.04) which is suitable for studying many of the biological organelles. The relative fluorescent quantum yield of the compound at the pH 6.0, 6.5, 7.0, and 7.5 are 0.529, 0.551, 0.767, and 0.814, respectively. On account of the complexity of the intracellular environment, an additional examination of the probe was performed to determine whether other ions were potential interferents. To investigate this phenomenon, metal ion selectivity assays were performed while keeping the other experimental condition unchanged at pH 6.63. Fluorescence enhancement of **1-H** (5 μM) was not observed upon the addition of a large excess 200 equiv (1 mM) of biologically relevant metal ions, i.e., Na^+, K^+, and Ca^{2+} and 20 equiv of excess of several metal ions (Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}). In presence of 200 times excess of various ions (1 mM) like Na^+, K^+, and Ca^{2+} together with **1-H**, almost no adverse effect on intensity was observed (Figure S4, see the Supporting Information). Also in the case of various metal ion mixtures [e.g., ($Fe^{3+} + Mn^{2+}$), ($Cu^{2+} + Zn^{2+}$), ($Fe^{3+} + Mn^{2+} + Cu^{2+} + Zn^{2+}$), and ($Na^+ + K^+ + Ca^{2+} + Fe^{3+} + Mn^{2+} + Cu^{2+} + Zn^{2+}$)] together with **1-H**, an almost similar fluorescence enhancement was observed as shown by the free probe itself. We have also measured the pK_a values (Table S2, Supporting Information) in presence of these metal ions and found that pK_a of **1-H** does not change with the interferences of metal ions. Those results imply that **1-H** can selectively measure pH in the presence of various

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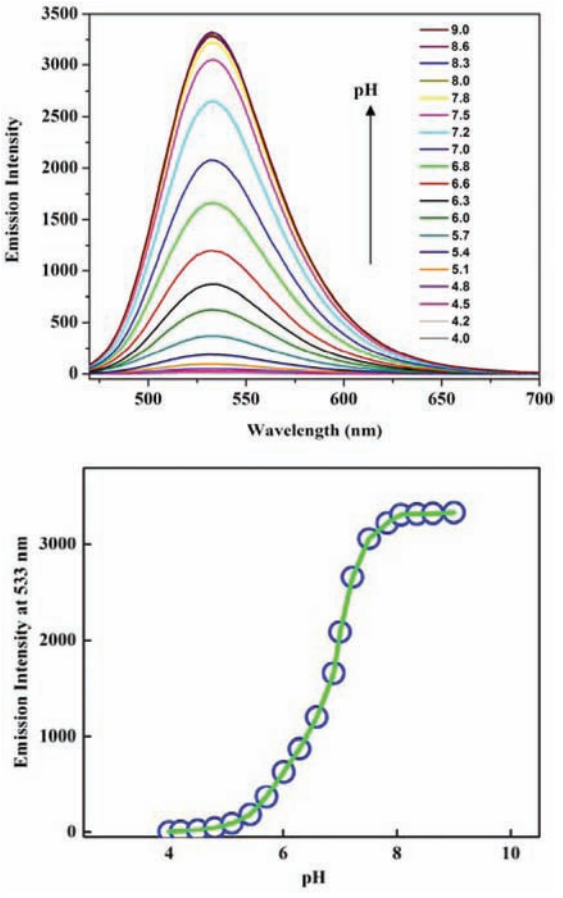
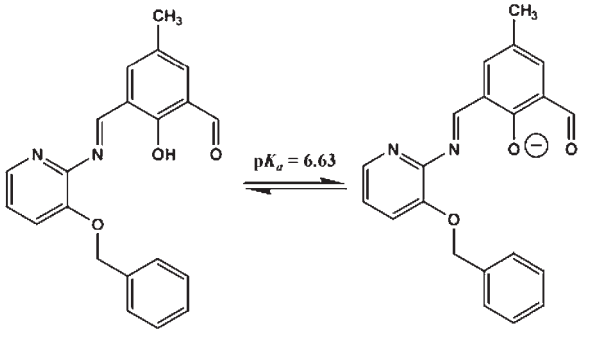


Figure 2. (a) Fluorescence spectra of **1-H** in various pH values (4.0−9.0). (b) Fluorescence response of **1-H** to pH variation at 25 °C in Britton−Robinson buffer.

metal ions usually present in biological systems, and therefore, **1-H** might be considered as a selective fluorescent probe for pH sensing. It is also noteworthy that the effect of such metal ions on pH measurement is negligible, particularly when considering that the concentrations used for the experiment were significantly higher than those present in the intracellular environment.

Scheme 1. Formation of **1** (Phenoxide) from **1-H** in Higher pH



The mode of protonation/deprotonation of **1-H** with pH was investigated by spectrophotometric titration at 25 °C in Britton−Robinson buffer. Figure 3 illustrated a typical UV−vis titration curve of **1-H** as a function of pH. The absorption intensity of **1-H** at 350 nm gradually decreased, accompanied by the formation of a new absorption peak at 430 nm, as the pH was increased stepwise from 5.0 to 8.0. The extinction coefficients (430 nm) of the compound are gradually enhanced to 0.26, 0.30, 0.39, 0.70, 0.94, and 1.22 × 10^4 M^{-1} cm^{-1} when the pHs are increased to 5.01, 5.50, 6.01, 6.49, 7.00, 7.52, and 8.02, respectively. The observed absorption maximum at 350 nm is attributed to the hydrogen-bonded phenolic form of **1-H**.[9] Intensity of the absorption band at 430 nm increases due to the formation of **1** (phenoxide) with an increase in pH, i.e., the increase in extinction coefficient at 430 nm, with a concomitant appearance of isosbestic points at ca. 380 nm. These phenomena illustrated the transformation from free **1-H** to **1** species. This phenomenon is further supported by the ^{1}H NMR data of **1** where the signal for −OH (at ca. δ = 15.16 ppm) was absent (Figure S5, Supporting Information). The fluorescent and absorption studies of the compound are reversible with the change in pH which supports in the favor of the stability of the compound. The fluorescence intensity of **1-H** at 533 nm was plotted against [H^+] to elucidate the binding stoichiometry of the **1** (phenoxide) with H^+. The fluorescence response fits to a Hill coefficient (Figure S6, see the Supporting Information) of **1** (1.08113). It is consistent with the formation of a 1:1 stoichiometry for the **1** (phenoxide)-H^+ species. This result implies that only one proton plays a crucial role in the protonation−deprotonation phenomenon.

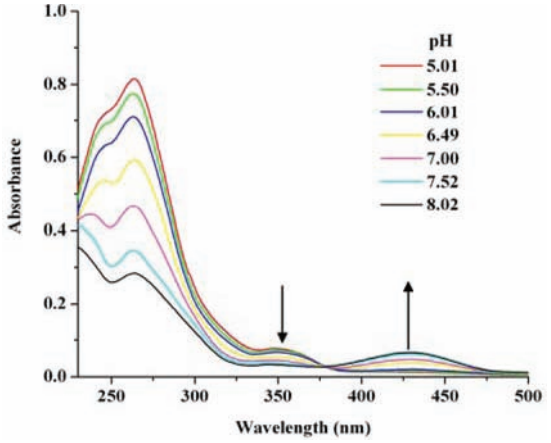


Figure 3. UV−vis absorption of **1-H** (5 μM) to pH variation at 25 °C in Britton−Robinson buffer.

To gain additional understanding of the excited state character of **1-H** and **1**, DFT calculations were performed using Dmol^3 code[11] in the framework of a generalized-gradient approximation (GGA) (Figure 4). The electronic

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densities of both the HOMO and the LUMO of the probe **1-H** appear mainly at the 2-hydroxy-5-methyl-3-(pyridin-2-yliminomethyl)benzaldehyde unit. This finding suggests that the "2-hydroxy-5-methyl-3-(pyridin-2-yliminomethyl)-benzaldehyde" moiety functions as the fluorophore. In the optimized structure of **1** (Figure S7, Supporting Information), the density of the HOMO of **1** has a similar distribution as that of the **1-H**, whereas the DFT calculations provide stabilization of the LUMO energy level of **1** relative to that of **1-H** (Figure S8, Supporting Information). It is postulated that the HOMO−LUMO transition corresponds to the emissive $\pi-\pi^*$ excited state as observed in bodipy (BODIPY = 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) derivatives.[12] Such stabilization of the electronic densities and increase of the structural rigidity of **1** upon deprotonation of **1-H** could be attributable to red shift.

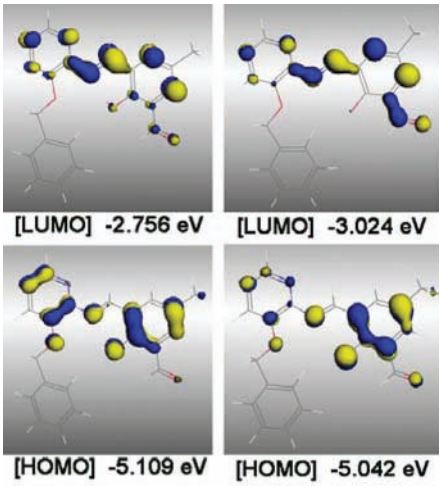

Figure 4. Molecular orbitals plot of **1-H** $[C_{21}H_{17}N_2O_2(OH)]$ (left) and **1** $[C_{21}H_{17}N_2O_2(O^-)]$ (right).

We applied the probe **1-H** to human cervical cancer cell, HeLa, and A375 human melanoma cells to examine whether it can work in biological systems. The extracellular pH was changed by the addition of 5 mM KOH or 50 mM HCl to DMEM, and the responses of intracellular pHs were monitored. The cells were incubated with the probe, **1-H** (5 mM) for 20 min at 25 °C at different pH (5.0, 6.0 and 6.6). The distribution of the probe within the cells was observed by fluorescence microscopy following excitation at ~440 nm (Figure 5). In addition, 5 μM of **1-H** did not show significant cytotoxic effect (Figure S9, Supporting Information) on both the cells for at least up to 6 h of its

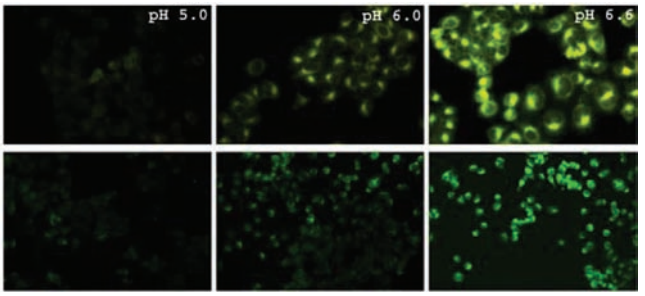

Figure 5. Fluorescence images of HeLa (upper row) and A375 (lower low) cells with concomitant increase in pH (5.0, 6.0, and 6.6) incubated with 5 μM of **1-H** for 20 min at 25 °C in DMEM. The extracellular pH was changed by the addition of 5 mM KOH or 50 mM HCl to DMEM, and the responses of intracellular pHs were monitored.

treatment though there was significant cytotoxicity for higher doses after 6 h onward. These results indicate that **1-H** is an efficient candidate for monitoring changes in intracellular pH under biological condition.

In conclusion, a newly designed probe, 3-[(3-benzyloxypyridin-2-ylimino)methyl]-2-hydroxy-5-methylbenzaldehyde (**1-H**) behaves as a highly selective fluorescent pH sensor in a Britton−Robinson buffer at 25 °C. It shows a 250-fold increase in fluorescence intensity within the pH range of 4.2 to 8.3 with a pK_a value of 6.63 which is appropriate for the biological organelles. This increase is accounted by the formation of **1** (phenoxide) ion from the less fluorescent phenolic **1-H** in the high range of pH. It is highly convenient for biological application as both wavelengths (λ_{ex} = 440 nm and λ_{em} = 533 nm) are not in the UV region, and the fluorescence intensities are almost unaffected by the biologically relevant (Na^+, K^+, Ca^{2+} etc.) and other metal (Cr^{3+}, Mn^{2+}, Fe^{3+}, etc.) ions. Furthermore, we have demonstrated the significance of **1-H** by monitoring intracellular H^+ within human cervical cancer cell, HeLa, and A375 human melanoma cells.

Acknowledgment. Financial assistance from the Department of Science and Technology (DST), New Delhi (project no. SR/S1/IC-37/2008) is gratefully acknowledged.

Supporting Information Available. Experimental section, Figures S1−S9, Tables S1 and S2, X-ray crystallographic details, CIF of **1-H**. This material is available free of charge via the Internet at http://pubs.acs.org.

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