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Fluorescent nucleosides with 'on–off' switching function, pH-responsive fluorescent uridine derivatives

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ABSTRACT

We synthesized various pH-responsive fluorescent deoxyuridine derivatives (**1a**–**g**). These fluorescent nucleosides exhibited distinctive fluorescence at 470–600 nm in aqueous solvents containing methanol only at acidic to neutral pH values. In particular, **1f** exhibited strong fluorescence only at pH range of 3.1–7.2 with a pK_a of 6.1. Such pH-sensitive fluorescent nucleosides can be used as 'on–off' fluorescence switch for monitoring pH change in biological systems, particularly for cancer cell detection.

Monitoring intracellular pH is important for better understanding of physiological and pathological processes in living cells. Many cellular events such as cell growth,[1] apoptosis,[2] endocytosis,[3] ion transport,[4] and many other cellular processes involve protonation and deprotonation of biomolecules with accompanying small pH changes in the microenvironment and various methods for monitoring pH in a cell have been proposed. Among these, fluorescent probes are very attractive because they are simple, highly sensitive, non-invasive, and nondestructive to cells. Actually, many pH-responsive fluorescent molecules have been developed.[5] Particularly, fluorescent molecular 'on–off' switch to detect acidic pH sites is very important for detecting cancer cell (pH *ca.*6). While several pH-sensing fluorescent molecules are known,[5] pH-responsive fluorescent nucleosides, to our knowledge, have not been reported. To date, numerous efforts to impart useful fluorescence features upon non-emissive natural nucleobases have been reported.[6] Many previous approaches involved the linking of natural nucleobases to fluorescent aromatic or heteroaromatic chromoph-

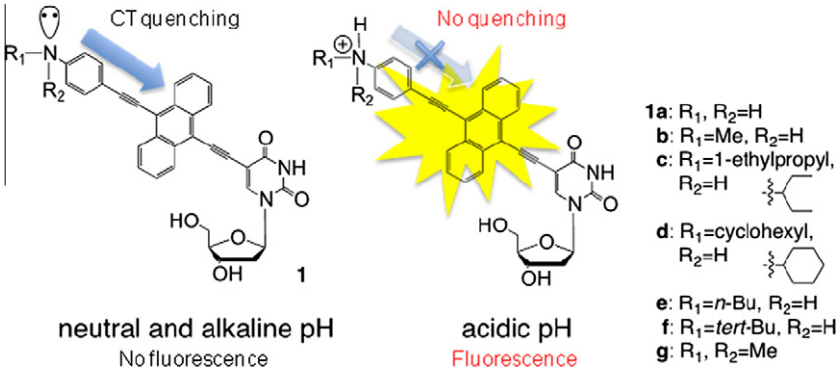

Figure 1. Structures of fluorescent pH-responsive nucleosides.

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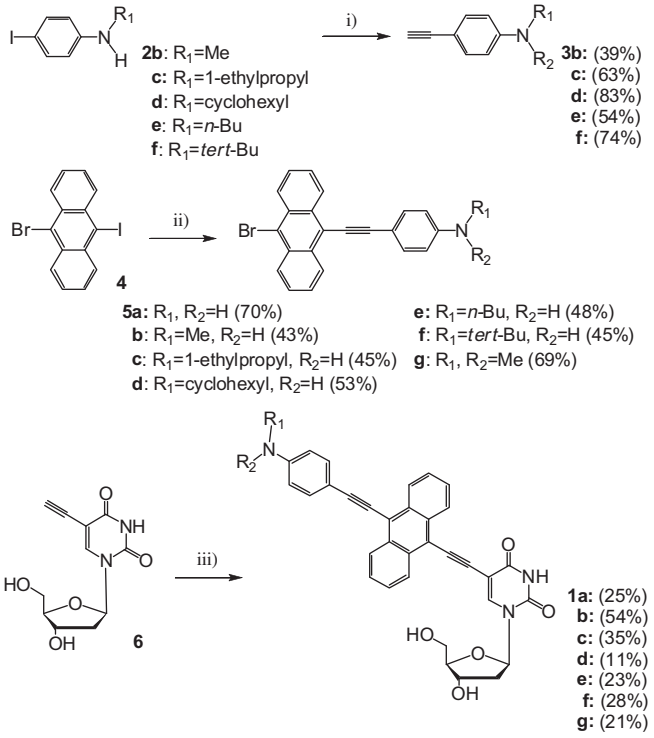

Scheme 1. Reagents and conditions: (i) trimethylsilyl acetylene, $Pd(PPh_3)_4$, TEA, THF, rt, 3 h, then TBAF, THF, rt, 20 min; (ii) **3b**–**f**, $Pd(PPh_3)_4$, CuI, *i*-Pr_2NH, toluene, rt, 12 h; (iii) **5a**–**g**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, 80 °C, 24 h.

Table 1
Physical properties of pH-responsive fluorescent nucleosides **1a**–**g**

Compounds	pK_a	UV λ_{max} (nm)			
		Acidic pH (<3.5)		pH (>5.0)	
1a	3.9	441	468	453	476
b	3.7	442	470	457[a]	480
c	3.7	445	472	457[a]	490
d	3.9	444	471	458[a]	485
e	3.5	444	471	459[a]	485
f	6.1	444	470 (pH <6.0)	458[a]	476 (pH >7.0)
g	3.0	440	468	440	478[a]

[a] Shoulder.

ores via an ethynyl linker[6] or by the direct attachment of fluorescent chromophores to natural nucleobases.[7] In fact, various 5-arylethynylated uracil derivatives have been reported.[6] In this study, we have designed highly pH-sensitive fluorescent 2′-deoxyuridine derivatives **1** that are π-conjugated with anthracene chromophore containing electron donating anilino group through an ethynyl linker. By changing the substituent of the *N*-alkyl group on the aniline moiety, a new family of fluorescent pH probes with tunable pK_a values were designed. The fluorescent switching function may involves a pH-dependent photoinduced electron transfer (PET) from aniline group to anthracene chromophore as shown in Figure 1.[8] This is because the unprotonated amines such as aniline are efficient electron-transfer quenchers of the photoexcited anthracene.[8] In contrast to our previously reported base-discriminating fluorescent (BDF) nucleosides,[6c,9] which show increasing or decreasing emission intensity at fixed wavelengths, the pH-

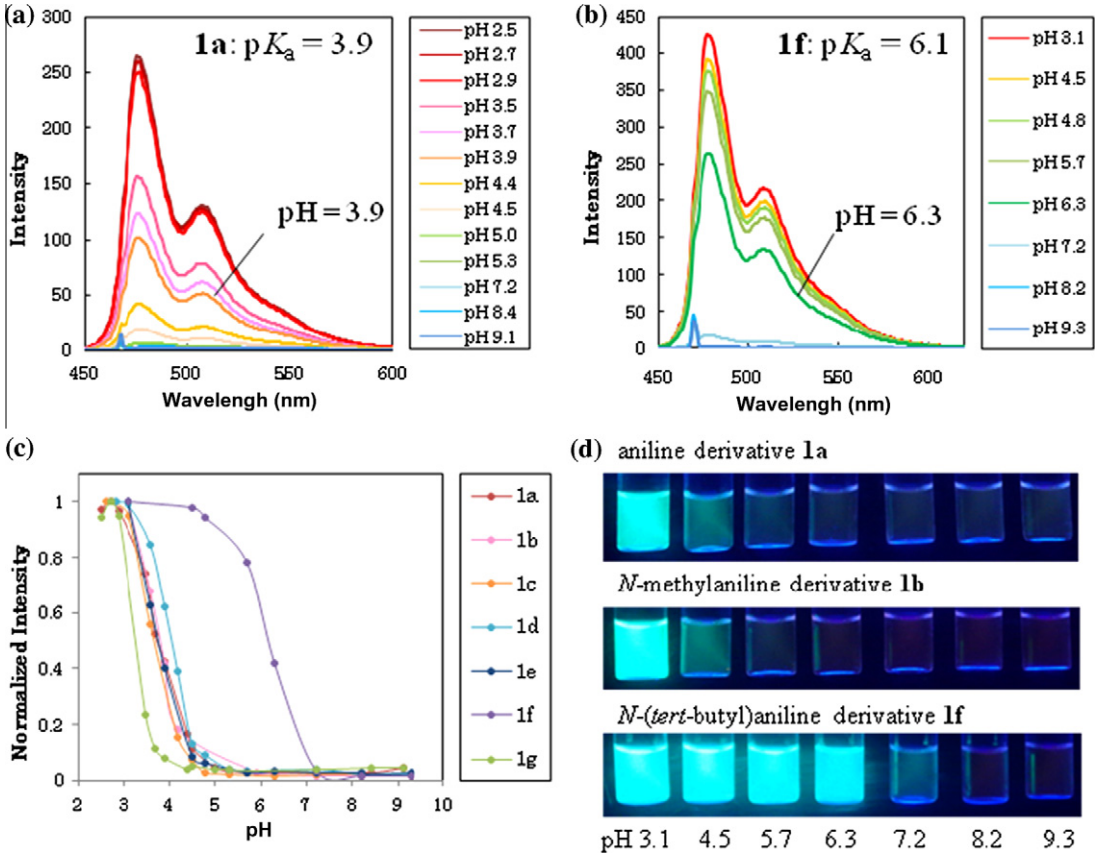

Figure 2. Fluorescence spectra of (a) **1a** (2.5 μM) and (b) **1f** (2.5 μM) at various pHs (2.5–9.3) in aqueous solvents containing methanol (H_2O:MeOH:DMF = 50:49:1). (c) Fluorescence response of **1a**–**g** versus pH as measured by fluorescence plate reader in aqueous solvents containing methanol (H_2O:MeOH:DMF = 50:49:1). (d) Fluorescence color image of **1a**, **1b** and **1f** at various pHs. The sample solutions were illuminated with 365 nm transilluminator.

dependent fluorescent nucleosides described here indicate pH changes in the local environment by incident appearance of fluorescence. These newly developed 'on–off' switching fluorescent nucleosides can be used for monitoring pH in the surrounding microenvironment of biological organelles and nucleic acids as well as specific acidic sites (pH *ca*.6) in a cancer cell.

The synthetic route of pH-responsive fluorescent nucleosides, **1a–g**, is outlined in Scheme 1. Secondary arylamines **2b–f** were prepared from 1,4-diiodobenzene according to the protocol of Fukuyama et al.[10] The palladium-catalyzed Sonogashira cross-coupling reaction[11] of **2b–f** with TMS-acetylene followed by deprotection with TBAF yielded compounds **3b–f**. The second Sonogashira coupling reaction of 9-iodo-10-bromoanthracene **4** with corresponding 4-ethynylaniline derivatives **3b–f** afforded bromoanthracene derivatives **5b–f**, respectively. Compounds of general structure **5** were then coupled with 5-ethynyl-2′-deoxyuridine **6** using $Pd(PPh_3)_4$ to yield pH-responsive fluorescent nucleosides **1b–f**. Compounds **1a** and **1g** containing anilino and *N,N*-dimethylanilino moieties were also prepared from commercially available 4-ethynylaniline and 4-ethynyl-*N,N*-dimethylaniline, respectively, by a similar route.[12]

The photophysical properties of the newly synthesized nucleosides **1a–g** were examined. Initially, we measured the fluorescent spectra of the aniline derivative **1a**, which has no substituent in the *N*-alkyl group at pH ranging from 2.5 to 9.1. As shown in Figure 2a, compound **1a** showed a pH-sensitive fluorescence emission at acidic pH range. Upon excitation of **1a** at 470 nm with acidic pH (<4), a strong fluorescence emission at 470–600 nm was observed. In contrast, at increasing pH, the fluorescence intensities rapidly decreased, and finally a very weak emission was observed in neutral and alkaline pH (>5) region. The pK_a of **1a** was obtained from the change in fluorescence intensities as a function of pH using the Henderson–Hasselbalch equation,[13] $\log[(F_{max} - F)/(F - F_{min})] = pH - pK_a$, where F is the observed fluorescence intensity at a fixed wavelength and F_{max} and F_{min} are the corresponding maximum and minimum intensities, respectively, that yielded pK_a value of 3.9. Since aniline derivative **1a** exhibited highly sensitive fluorescence emission, other *N*-alkylaniline derivatives, which are estimated to have different pK_as,[14] were examined. As a result, most other *N*-alkylated aniline derivatives, **1b–e** and **1g**, exhibited strong fluorescence emissions below pH 5 with pK_a of 3.7, 3.7, 3.9, 3.5 and 3.0, respectively (Table 1). Interestingly, in the case of *N*-(*tert*-butyl)aniline derivative **1f**, the fluorescence emission was observed at a higher pH range than that of other nucleosides. As shown in Figure 2b, **1f** exhibited more than a 250-fold increase in the fluorescence intensity within the pH range of 4.8–8.2 with a pK_a of 6.1. We also measured the fluorescence response of the newly synthesized fluorescent nucleosides **1a–f** to pH variation by means of fluorescence plate reader. As indicated in Figure 2c, these fluorescent nucleosides showed a sigmoidal correlation between fluorescence intensity and pH. These results indicated that **1f** emits strong fluorescence at higher pH than other nucleosides **1a–e** and **1g** as visualized by the fluorescence color image shown in Figure 2d.

The pH-dependent fluorescent behavior was attributable to the protonation/deprotonation equilibrium of the aniline moiety. The mode of protonation/deprotonation of **1a–g** with varying pHs was also investigated by UV spectrometric titration. Figure 3a shows a change of UV–vis absorption spectra of **1a** at various pHs. The absorption intensity of **1a** at 441 and 468 nm gradually red-shifted to 453 and 476 nm, respectively, as pH increased from 2.5 to 9.1. A similar red-shift of the absorption peak by increasing pH was also obtained from *N*-(*tert*-butyl)aniline derivative **1f** (Fig. 3b) and other *N*-alkylaniline derivatives **1b–d** (Table 1). Such

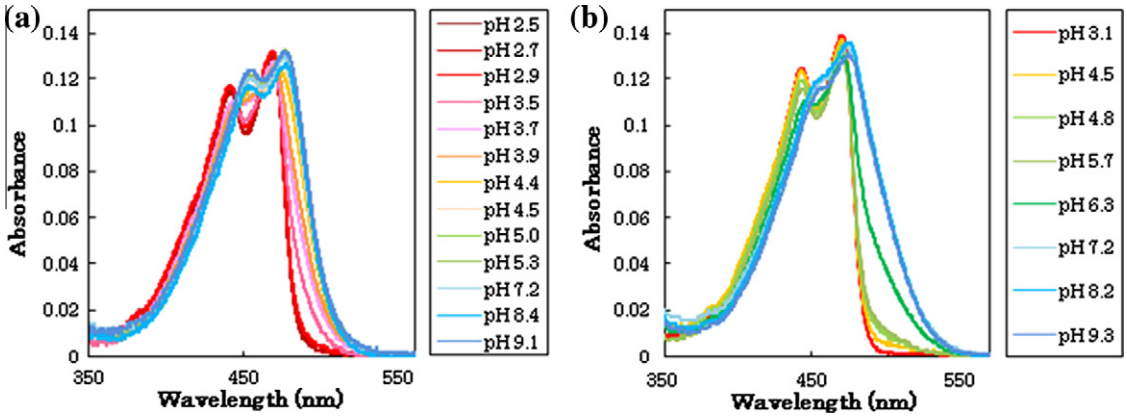

Figure 3. UV–vis absorption of (a) **1a** (2.5 μM) and (b) **1f** (2.5 μM) at various pHs in aqueous solvents containing methanol (H_2O:MeOH:DMF = 50:49:1).

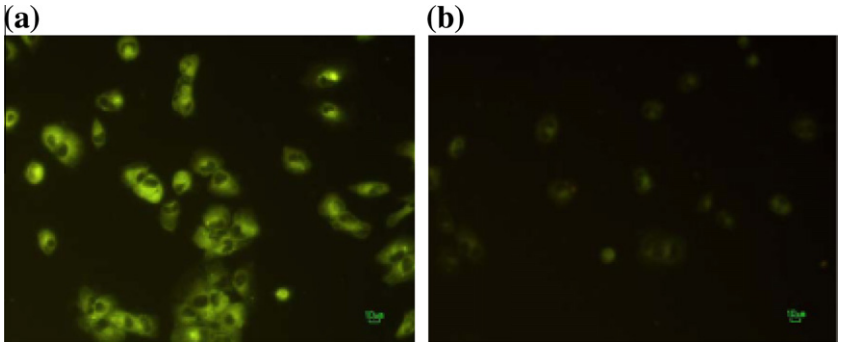

Figure 4. The living cultured cells were stained with (a) **1f** and (b) **1b**. Huh-7 cells on a chamber slides were incubated with 1 μM of **1f** and **1b** in RPMI medium supplemented with 10% FBS at 37 °C for 2 h. After washing three times with phosphate buffered saline (PBS), fresh medium was added and cells were observed by fluorescence microscope.

red-shift of the UV absorption suggests the formation of free amine from the protonated anilinium ion with increasing pH. Due to the efficient photo-induced electron transfer (PET) from the aniline moiety to the anthracene fluorophore, almost no fluorescence emission was observed in a neutral to basic pH region.[8] The fluorescence and absorption behaviors of these compounds are reversible with changing pH, supporting a protonation–deprotonation process.

Next, a preliminary study of the newly synthesized fluorescent nucleosides in living cells was carried out by means of fluorescence microscopy. We treated human hepatoma cell line Huh-7 with structurally similar two nucleosides, *N*-methylanilino derivative **1b** (pK_a 3.9) and *N*-(*tert*-butyl)anilino derivative **1f** (pK_a 6.1), at 37 °C for 2 h. As shown in Figure 4, *N*-(*tert*-butyl)anilino derivative **1f** was penetrated into cell membranes and exhibited strong green fluorescence in the cytosol. In contrast, extremely weak fluorescence emission was observed when the cell was treated with *N*-methylanilino derivative **1b**. We observed a large difference in fluorescence behavior between these two fluorescent nucleosides **1b** and **1f**, which is the indication of the capability of **1f** to discriminate acidic site of human hepatoma cell.

In conclusion, we have succeeded in the design and synthesis of highly pH-sensitive fluorescent uridine derivatives **1a–g**. In particular, *N*-(*tert*-butyl)aniline derivative **1f** emitted strong fluorescence at 470–600 nm at pH below *ca*.6.8 with a pK_a of 6.1. The result indicated that newly synthesized fluorescent nucleoside **1f** can be used for monitoring pH change under physiological conditions. These newly synthesized pH-dependent fluorescent nucleosides can be used as fluorescence 'on–off' switch for probing acidic sites in a cell.

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12. Spectroscopic data for **1a**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.24 (ddd, J = 4.4, 6.2, 13.4 Hz, 1H), 2.33 (m, 1H), 3.69 (m, 1H), 3.77 (m, 1H), 3.87 (m, 1H), 4.36 (m, 1H), 5.34 (d, J = 4.4 Hz, 1H), 5.37 (m, 1H), 5.80 (s, 2H), 6.21 (m, 1H), 6.68 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 8.6 Hz, 2H), 7.73–7.77 (complex, 4H), 8.62-8.71 (complex, 4H), 8.74 (s, 1H), 11.9 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 40.3, 60.7, 69.6, 83.8, 85.1, 87.7, 89.5, 95.7, 98.5, 105.7, 107.9, 113.8 (×2), 116.2 (×2), 118.7 (×2), 126.9 (×2), 127.3 (×2), 127.5 (×2), 130.7 (×2), 131.0 (×2), 133.1 (×2), 143.8, 149.5, 150.3, 161.7; HRMS (ESI) m/z 566.1691 calcd for $C_{33}H_{25}N_3O_5Na$ [M+Na]$^+$, found 566.1707.
Spectroscopic data for **1f**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.37 (s, 9H), 2.24 (ddd, J = 4.4, 6.2, 13.3 Hz, 1H), 2.33 (m, 1H), 3.69 (m, 1H), 3.77 (m, 1H), 3.87 (m, 1H), 4.36 (m, 1H), 5.35 (d, J = 4.2 Hz, 1H), 5.38 (m, 1H), 6.03 (s, 1H), 6.21 (m, 1H), 6.83 (d, J = 8.8 Hz, 2H), 7.57 (d, J = 8.8 Hz, 2H), 7.73–7.77 (complex, 4H), 8.62–8.71 (complex, 4H), 8.75 (s, 1H), 11.9 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 29.2 (×3), 40.3, 50.3, 60.7, 69.6, 84.1, 85.0, 87.7, 89.5, 95.8, 98.5, 105.6, 107.4, 114.2 (×2), 116.3 (×2), 118.7 (×2), 126.9 (×2), 127.3 (×2), 127.5 (×2), 130.7 (×2), 131.0 (×2), 132.8 (×2), 143.8, 148.8, 149.5, 161.7; HRMS (ESI) m/z 622.2318 calcd for $C_{37}H_{33}N_3O_5Na$ [M+Na]$^+$, found 622.2307.
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