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9-Aryl-9-xanthenols: a convenient platform for the design of fluorimetric and colorimetric pH indicators†

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Abstract

In aqueous and alcohol solutions, colorless and non-fluorescent derivatives of 9-aryl-9*H*-xanthen-9-ol equilibrate with brightly colored and fluorescent 9-arylxanthylium cations, thus offering a convenient platform for the design of dual-mode indicators for emission and absorption-based pH measurements. The position of the prototropic equilibrium depends only on the hydronium ion concentration and is not affected by general acids or other ions. Furthermore, the equilibrium equivalence point can be readily adjusted by introducing substituents in the xanthenol core. As dehydroxylation of 3,6-dialkoxy-9-(*o*-tolyl)-9-xanthenol occurs at pH = 6.5, indicators of this type are well suited for biological applications as illustrated by *in vitro* cell culture studies with NIH 3T3 cells.

Introduction

The maintenance of specific intracellular pH is crucial for the proper functioning of the cellular machinery. The hydronium ion concentration is maintained within very strict limits by a complex system of buffering mechanisms.¹ The normal cytosolic concentration of hydronium ions in mammalian cells is estimated at ca. 4×10^{-8} mol L⁻¹. Slight variation in this value can impair enzyme activities,² synaptic transmission,³ and appears to play a role in Alzheimer's,⁴ chronic granulomatous disease,⁵ mucopolipidosis type IV,⁶ as well as cardiopulmonary and neurological conditions.⁷ Furthermore, abnormal alteration in cytosolic and vesicular acidity has been associated with cancer.⁸ On the other hand, some acidic organelles maintain a pH range of about 4.5–6.5 for their proper function.^{1,9} Among the various techniques used for the measurement of cellular pH such as NMR, microelectrode, and absorbance spectroscopy, fluorescence spectroscopy is attractive because it is non-invasive, has greater analytical sensitivity and specificity, and offers good spatial and temporal resolution.¹⁰

The design of the majority of modern fluorescent pH indicators is based on three acidity-dependent phenomena. The first class of indicators, which includes the popular 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF),¹¹ relies on the pH-dependent emission intensity of fluorescein-derived fluorophores.¹² Below pH 5 fluorescein forms a weakly fluorescent charge-neutral species, which also equilibrates with a non-fluorescent spirolactone isomer. Between pH 5 and 6.5 the monoanionic (carboxylate) form of the dye,

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which fluoresces with a quantum yield of $\Phi_{\text{fl}} = 37\%$, predominates, whereas at higher pH the equilibrium is shifted towards the highly emissive dianion ($\Phi_{\text{fl}} = 93\text{--}95\%$).¹³ The equivalence points of these equilibria, as well as the absorption and emission maxima of the associated species, can be altered by modifying the xanthene core of the dye.^{10,14} Rhodamine-based pH indicators exploiting analogous spirolactam–amide equilibria have also been explored.¹⁵ The second group of fluorescent pH indicators utilizes a photo-induced electron transfer (PET)-switching mechanism to modulate the emission intensity.^{10,16} Under neutral or basic conditions, PET to the fluorophore from an adjacent basic receptor moiety with a high-energy HOMO (usually an amine) quenches the emission. Protonation of the basic moiety lowers the HOMO energy and renders PET unfavourable, thus restoring the fluorescence. Unfortunately, the receptor HOMO energy is not only dependent on the protonation state but can be also influenced by other parameters, such as changes in solvent polarity or viscosity, as well as in the local electrostatic environment.¹⁷ Finally, the photophysical properties of the green fluorescent protein (GFP) and its analogs depend on the pH of the solution.¹⁸ Several variants of GFP and BFP have been developed to report real-time changes of pH in live cells.¹⁹ Limitations of currently available fluorescent pH indicators, especially their sensitivity towards environmental changes, have led to the continuous exploration of new design principles.²⁰

Here, we report a new platform for the design of fluorescent pH indicators, which is based on hydronium-ion-mediated reversible dehydroxylation of 9-aryl-9*H*-xanthen-9-ol (Scheme 1). The position of this equilibrium between colorless and non-fluorescent 9-aryl-9*H*-xanthen-9-ol and the brightly colored and fluorescent 9-arylxanthylium cation depends only on the hydronium ion concentration and is not affected by general acids or other ions. pH measurements using such indicators are less dependent on the environment and allow for both fluorescent and colorimetric readout.

Results and discussion

Triarylmethanol derivatives are known to form stable coloured cations in concentrated acids.²¹ Incorporation of xanthene fragments makes these cations highly fluorescent.²² Thus, the parent 9-phenyl-9-xanthenol (**1a**) is known to reversibly produce the fluorescent 9-phenyl-9-xanthylium cation (**2a**) in 25% H₂SO₄ (Scheme 1). The equivalence point (when [1a] = [2a]) was reported at pH = 1.0 ± 0.5.²³

We hypothesized that introduction of electron donating substituents in the 3- and 6-positions of the xanthenol ring would shift the equivalence point to biologically relevant pH values. To test this hypothesis, we prepared 3,6-bis-allyloxy-9-phenyl-9*H*-xanthen-9-ol (**1b**), which showed reversible formation of the xanthylium cation below pH 5 as judged by the increase in absorbance at 437 nm. The equivalence point for the **1b–2b** equilibrium is achieved at pC_{H+} = 4.46 ± 0.02²⁴ (Fig. 3). As *ortho*-substituents on the 9-aryl group are known to enhance the fluorescence quantum yield of xanthylium-based fluorophores,²⁵ we replaced the phenyl group in **1b** with the *o*-tolyl moiety (Scheme 1, **1c**). This substitution resulted in a shift of the indicator equivalence point to a higher pH value (Fig. 3). This observation demonstrates that the pH-dependent equilibrium of the indicator can be adjusted not only by altering the electronic properties of the xanthylium unit but also by structural changes. We believe that the increased steric congestion around tertiary alcohols thermodynamically favours the dehydroxylation reaction.

The synthesis of 9-aryl-9-xanthenols **1b**, **1c** and **6** started with 3,6-dihydroxy-9*H*-xanthen-9-one (**3**), which was prepared by acid-catalyzed cyclization of commercially available 2,2', 4,4'-tetrahydroxybenzophenone (Scheme 2).²⁶ Successive alkylation of the two hydroxy groups in **3** produced 9-xanthenone **5**, which was treated with *o*-tolylmagnesium bromide to

give the target compound 3-allyloxy-6-methoxy-9-(2-methylphenyl)-9*H*-xanthenol (**1c**) in 30% overall yield. Dialkylation of dihydroxyxanthen-9-one (**3**) with allyl bromide afforded 3,6-diallyloxy-9*H*-xanthen-9-one (**7**). The latter was converted to xanthenol **1b** by the reaction with phenylmagnesium bromide. In the presence of alcohols, 9-aryl-9-xanthenols are smoothly converted to the corresponding ethers. Thus overnight heating of **1c** in MeOH in the presence of a catalytic amount of AcOH quantitatively produced the methyl ether **6**.

The absorption spectrum of **1c** in MeOH shows a band with $\lambda_{\text{max}} = 275 \text{ nm}$ ($\log \epsilon = 4.14 \pm 0.016$). In a 20% CH_3CN –0.1 M HClO_4 solution, **1c** is completely converted into the 9-aryl-9-xanthylium cation **2c** as is evident from a strong characteristic band at $\lambda_{\text{max}} = 437 \text{ nm}$ ($\log \epsilon = 4.77 \pm 0.002$). The formation of cation **2c** was further confirmed by high-resolution mass spectrometry and NMR spectroscopy.²⁶ Comparison of ^1H and ^{13}C spectra of **2c** with that of **1c** shows a significant downfield shift of most resonances in cationic form.²⁶ In the proton spectrum the strongest deshielding influence of the cation formation is observed at positions 4 and 5 ($\Delta\delta \sim 1 \text{ ppm}$). Signals of the methoxy group and CH_2 of the allyloxy substituent are observed at higher frequencies in ^1H and ^{13}C spectra of cationic form (**2c**) of the indicator. This observation suggests significant participation of oxygen atoms of these substituents in charge delocalization. In the carbon spectrum the formation of the cation is most clearly illustrated by the downfield shift of the carbon at the 9-position (from 69.7 ppm in **1c** to 169.5 ppm in **2c**) and 3/6 (from ~ 160 to $\sim 173 \text{ ppm}$).²⁶ Solutions containing only **1c** ($\text{pH} > 12$) show no measurable fluorescence, while **2c** exhibits an intense emission band with $\lambda_{\text{max}} = 477 \text{ nm}$ ($\Phi_{\text{fl}} = 16\%$, Fig. 1b), a value that compares well with other substituted 9-phenylxanthylium ions.²⁷ The excitation spectrum maximum of **1c** coincides with the major absorption band at 437 nm (Fig. 1). It should be noted that reports by Wan, Yates, and Boyd have consistently shown that the emission from the parent 9-phenylxanthylium ion is quenched by water;²⁸ emission spectra of **2c**, however, were recorded in aqueous solutions.

The dehydroxylation reaction of 9-aryl-9-xanthenols is strongly facilitated by hydronium ions. At $\text{pH} < 3$, equilibration between xanthenol **1c** and xanthylium cation **2c** occurs virtually instantly, with the lifetime of the leuco-form of the indicator (**1c**) being below 100 ms. Rates of the dehydroxylation of xanthenol **1c** were therefore studied in acetate, biphosphate ion, TRIS, and bicarbonate ion buffers. The ionic strength of these solutions was kept constant at 0.1 M by adding sodium perchlorate as required. Accurate rate measurements were conducted by stopped-flow kinetic spectroscopy at $25 \pm 0.1 \text{ }^\circ\text{C}$ following the rise of absorbance at 437 nm. Rate measurements in buffered solutions were performed in a series of solutions of varying buffer concentration but constant buffer ratio. No appreciable buffer catalysis was detected. The observed pseudo-first-order rate constants (k_{OBS}) at various acidities of the solution are shown as the rate profile in Fig. 2.

The rate of the dehydroxylation of **1c** exhibits a strong dependence on the hydronium ion concentration, $k_{\text{H}^+} = 2073 \pm 67 \text{ M}^{-1} \text{ s}^{-1}$ (insert in Fig. 2). It is important to note that this reaction is only promoted by the specific acid (H_3O^+) but not by general acids, as evidenced by the absence of buffer catalysis. At $\text{pH} > 7$ the rate of **1c**–**2c** equilibration is virtually independent of the acidity of the solution leveling off at $\text{ca. } 7 \times 10^{-4} \text{ s}^{-1}$. It has to be noted that accurate rate measurements in this range become progressively difficult due to the very weak signal of the cation **2c**. This plateau region has two possible interpretations, the first of which is protonation of the xanthenol **1c** by water, followed by the loss of H_2O . This mechanism can be ruled out on the basis of the non-existent general acid catalysis by a much stronger acid (AcOH). The more probable mechanism of the uncatalyzed reaction is simple dissociation of **1c** to produce cation **2c** and a hydroxide ion.

Above pH 8, xanthenol **1c** has no significant absorbance beyond 350 nm but at lower pH the absorbance at 437 nm grows due to the increased fraction of xanthylium cation **2c** in the

equilibrium. This growth saturates at $\text{pH} < 5$, indicating complete conversion of the substrate into the cationic form **2c**. The equivalence point, where $[\mathbf{1c}] = [\mathbf{2c}]$, was determined by spectrophotometric titration to be at $\text{pC}_{\text{H}^+} = 6.57 \pm 0.02$ (Fig. 3).

Similar pH-dependent changes were observed in the emission spectrum (Fig. 4). Above $\text{pH} = 8$ the fluorescence of aqueous solutions of xanthenol **1c** is barely detectable, whereas at lower pH values an emission band at 477 nm grows in parallel with the acidity of the medium (Fig. 4).

The contrast of fluorescence indicators is defined by the ratio of the emission intensities between the “on” and “off” states, *i.e.*, the $F_{\text{max}}/F_{\text{min}}$ ratio of the probe.²⁹ Xanthenol **1c** shows a remarkable >1300-fold increase in fluorescence intensity between acidic ($\text{pC}_{\text{H}^+} = 3$) solutions and basic ($\text{pC}_{\text{H}^+} = 10.4$).

The normalized emission intensity of **1c** at 477 nm shows a smooth sigmoid dependence on the pC_{H^+} of the solution with an inflection point at $\text{pC}_{\text{H}^+} = 6.52 \pm 0.05$ (Fig. 5), which is in excellent agreement with the equivalence point obtained from spectrophotometric titrations. This observation further supports the assignment of the photochromic changes in aqueous solutions to the equilibrium between xanthenol **1c** and the xanthylium cation **2c**. It is interesting to note that at $\text{pH} < 3$, the emission spectrum of the xanthylium ion shows an additional small enhancement (Fig. 5). This phenomenon is also observed for the parent xanthylium ion **2a** but at much higher acidities ($H_0 = -3$, *ca.* 39% H_2SO_4 ^{28a}). This was originally explained by the reduction of effective water concentration in concentrated acids; however, in our case this enhancement is observed in very dilute solutions.

Given the near-neutral pK_a of xanthenol **1c**, we explored the utility of this indicator for the visualization of acidic compartments in cultured cells.²⁶ NIH 3T3 fibroblast cells were grown on glass cover slips and incubated with a 5 μM solution of xanthenol **1c** in serum-free DMEM (Dulbecco's modified Eagle's medium) for 1 h at 37 °C. Consistent with the lipophilic character, the dye readily crossed the plasma membrane and localized within cellular compartments, but showed negligible cytosolic fluorescence (Fig. 6A). To test whether the subcellular staining pattern coincides with acidic compartments, we performed a colocalization experiment in which cells were transfected with a plasmid encoding the red fluorescent protein RFP fused to Lamp1 for lysosomal targeting. Surprisingly, the compartments stained by xanthenol **1c** showed essentially no spatial correlation with lysosomes as illustrated by the red-green false colour overlay in Fig. 6B (right panel). In contrast, an analogous experiment with mitochondrial targeted red fluorescent protein (mito-dsRed) revealed a high degree of colocalization (Fig. 6C). At first, the observed mitochondrial localization of **1c** might appear unexpected; however, it is well known that cationic lipophilic compounds are sequestered by mitochondria, driven by the favourable mitochondrial membrane potential.³⁰ As a key component of the electron transport chain, protons are extruded from the mitochondrial matrix into the intermembrane space (IMS), resulting in a slightly basic matrix pH of 7.7 and a more acidic IMS pH of 6.8.³¹ According to Fig. 5, such a pH gradient would result in a more than 3-fold difference in emission intensity of **1c**, suggesting that the observed cellular staining predominantly originates from the dye localized in the IMS. Although there remains some uncertainty to what extent the equivalence point of the protonation equilibrium might shift to a higher pH if **1c** associates with membranes, the trend in fluorescence response would still favor the lower pH environment.

Conclusions

9-Aryl-9*H*-xanthen-9-ol derivatives represent a promising platform for the development of fluorescent pH indicators, which offer a number of beneficial features. First, the position of the equilibrium between the leuco- and the coloured/fluorescent form of the dye depends only on the concentration of hydronium ions. The equivalence point can be readily adjusted by introducing appropriate substituents. Given the apparent pK_a of 6.5 for indicator **1c**, we already identified a derivative that is well tuned towards a broad range of biochemical applications. Second, fluorescent xanthylium ions offer a high extinction coefficient coupled with bright emission, yielding a greater than 1300-fold fluorescence contrast compared to the neutral leuco-form. Furthermore, pH changes can be followed by both fluorescence and absorption measurements. Finally, the alkoxy groups and/or the 9-aryl moiety can be readily modified to change the lipophilicity or aqueous solubility of the dye and introduce linkers for conjugation to proteins and other biomolecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.
Reversible dehydroxylation of 9-aryl-9-xanthenols.

Scheme 2.

Synthesis of 9-aryl-9-xanthenols **1b**, **1c** and **6**. *Reagents and conditions*: (a) CH_3I , NaOH, BTEAC, toluene, reflux, 6 h, 57%; (b) allyl bromide, K_2CO_3 , acetone, reflux, 1 h, 64%; (c) *o*-tolylmagnesium bromide, rt, THF, 2 h, 80%; (d) AcOH (cat), MeOH, reflux, overnight, 95%; (e) allyl bromide, Cs_2CO_3 , DMF, overnight, 94%; (f) $PhMgBr$, rt, 2 h, THF, 77%.

Fig. 1.

(a) Absorption spectra of a 1.4 μM solution of **1c** in MeOH (black trace) and 0.1 M HClO_4 (blue trace). (b) Excitation ($\lambda_{\text{fl}} = 477$ nm, blue trace) and emission spectra ($\lambda_{\text{exc}} = 437$ nm, green trace) of the latter solution.

Fig. 2. Rate profile for the dehydroxylation of xanthenol **1c** in aqueous solutions at 25 °C. The insert illustrates the linear dependence of the rate on hydronium ion concentration.

Fig. 3. Absorbance of 27.5 μM aqueous solutions of xanthenols **1c** (triangles) and **1b** (circles) at different acidities.

Fig. 4.
Emission spectra of 1.4 μM aqueous solutions of xanthenol **1c** at different acidities.

Fig. 5. Fluorescent emission ($\lambda_{\text{exc}} = 437 \text{ nm}$) of $1.4 \mu\text{M}$ xanthanol **1c** aqueous solutions at various hydronium ion concentrations.

Fig. 6.

Fluorescence micrographs of NIH 3T3 fibroblast cells showing (A) the subcellular distribution of xanthenol **1c** (A) and colocalization with red fluorescent protein markers (red) targeted to lysosomes (B) or mitochondria (C). Cells were transfected either with Lamp1-RFP (lysosomes) or mito-dsRed (mitochondria), followed by incubation with 5 μM of xanthenol **1c** for 1 h. Areas of colocalization appear yellow in the false colour overlays. Scale bar: 10 μm .