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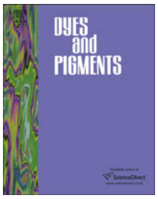

The synthesis and photophysical properties of halide sensitive acridizinium dyes

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ABSTRACT

A series of acridizinium (benzo[*b*]quinolizinium) salts containing methoxy, fluoro and chloro substituents were synthesized and their spectroscopic properties and sensitivity towards halide quenching in water were determined. The salts have substantially improved properties over the commonly employed quinolinium and acridinium fluorophores for the sensing of bromide and iodide ions. Of the substituted acridiziniums investigated, 8,10-difluoro-acridizinium bromide has the best combination of properties as a bromide or iodide sensor.

1. Introduction

There has been a considerable effort towards the development of halide sensing probes for both biological and industrial applications [1]. For this reason, fluorophores which undergo dynamic fluorescence quenching with halides have attracted considerable interest [2]. Quinolinium fluorophores display strong collisional quenching with halide ions and have been used to measure iodide, bromide and chloride concentrations in a variety of solutions with great success [2]. We have recently expanded the scope of application for quinolinium fluorophores via integration of their halide sensitivity into sensors for biologically important polyanions (*i.e.* glycosaminoglycans) [3]. Although quinolinium dyes work very well in many *in vitro* applications, it would be beneficial to have sensing-fluorophores with larger extinction coefficients at longer wavelengths to reduce experimental difficulties with applications in biological samples.

Many fluorophores with longer wavelength excitation have been explored as halide sensors [2]. Acridinium dyes show promise as they are quenched by the heavier halides (*i.e.* bromide and iodide). However, these heterocycles are unstable and degrade to non-fluorescent or halide insensitive products in less than 10 min under physiological conditions [4]. The long wavelength dye lucigenin [5] has the best spectroscopic properties as a chloride sensor ($\lambda_{ex} = 455$ nm, $\varepsilon = 7400\ \mathrm{M^{-1}\,cm^{-1}}$), but its size, challenging functionalization and similar problems with stability as the acridiniums preclude its use in our sensing applications [3,4]. As part of our ongoing effort to discover improved fluorescent halide sensors, we have evaluated a series of acridizinium (benzo[*b*]quinolizinium) fluorophores for their potential halide sensitivity.

Acridizinium salts (**1**, Fig. 1) were first described by Bradsher in 1955 and can be prepared by the aromatic cyclodehydration of pyridine-2-carboxyaldehydes and benzyl bromides [6]. These salts have been predominantly studied for their nucleophilic ring-opening, leading to pharmaceutically interesting structures [7,8]. Only recently, acridizinium derivatives were reported as an interesting class of fluorophores that can be applied as water-soluble pH sensors [9] and as biologically stable DNA and protein “light-up” probes [10–13]. Due to their structural and spectroscopic similarities to quinolinium and acridinium fluorophores we anticipated that acridizinium salts (Fig. 1) could be a potentially interesting class of halide sensors that would be suitable for use under physiological conditions. However, current knowledge about their dynamic quenching by halide ions is limited to a 1979 study by Bendig et al. performed in methanol, where quenching was observed for Cl^-, Br^- and I^- ions [14]. This data is difficult to translate to an aqueous environment where dynamic quenching will be less strong due to solvation of the halides. Herein we report the synthesis, photophysical properties, and dynamic quenching in water of a series of acridizinium fluorophores.

2. Experimental

2.1. Instrumentation and materials

All chemicals were purchased at Sigma Aldrich and used without further purification. Proton and carbon NMR spectra were

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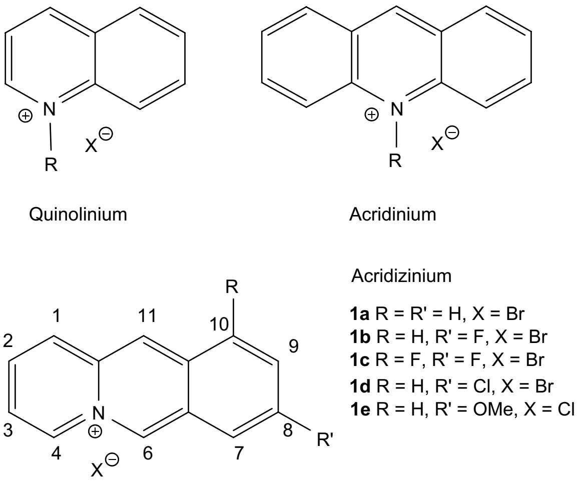


Fig. 1. Structures of common halide sensitive fluorophores and acridizinium salts **1a**–**e**.

recorded on a Varian Mercury 300 MHz NMR spectrometer. All spectroscopic measurements were performed with quartz cuvets using doubly distilled deionized water as a solvent. Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer, whereas fluorescence emission spectra were measured using a Fluorolog-3 spectrofluorometer. Fluorescence lifetimes were measured using an IBH system equipped with a 370 nm NanoLED pulsed diode source. Electrochemical measurements were made on a BAS-CV50 electroanalyzer utilizing a glassy carbon working electrode, platinum wire counter electrode, and an Ag/AgCl reference electrode. All compounds were dissolved in doubly distilled deionized water and potassium nitrate (1 M) was used as the supporting electrolyte.

2.2. Synthesis

The synthesis of acridizinium salts **1a**–**e** was adapted from a literature procedure [15]. Pyridine-2-carboxaldehyde (10 mmol) and the corresponding benzyl bromide (10 mmol) were refluxed in methanol (20 ml) for 3 h. After *in vacuo* removal of the solvent, the residue was treated overnight with concentrated hydrobromic acid at 80 °C (**1a**–**d**) or concentrated hydrochloric acid at 50 °C for 5 h (**1e**). *In vacuo* removal of the solvent lead to a solid which could be recrystallized from ethanol.

2.2.1. Acridizinium bromide (1a) [6]

Obtained in 75% yield as yellow crystals, m.p. 240–242 °C (dec). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 10.30$ (s, 1H), 9.23 (d, $J = 7.0$ Hz, 1H), 9.14 (s, 1H), 8.52 (d, $J = 8.7$ Hz, 1H), 8.46 (d, $J = 8.5$ Hz, 1H), 8.38 (d, $J = 8.6$ Hz, 1H), 7.98–8.15 (m, 3H), 7.90 (t, $J = 6.7$ Hz, 1H); ^{13}C NMR (300 MHz, CD_3OD): $\delta = 139.8$, 138.1, 136.3, 134.8, 134.0, 131.2, 130.9, 128.0, 127.1, 127.0, 126.7, 125.1, 122.5. Exact mass (ESI-MS+) calcd for [$C_{13}H_{10}N$]: 180.0808, found: 180.0807.

2.2.2. 8-Fluoro-acridizinium bromide (1b)

Obtained in 45% yield as yellow crystals, m.p. 242–244 °C (dec). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 10.32$ (s, 1H), 9.31 (d, $J = 6.9$ Hz, 1H), 9.21 (s, 1H), 8.56 (d, $J = 8.9$ Hz, 1H), 8.46 (dd, $J = 9.4$, 5.2 Hz), 8.06–8.14 (m, 2H), 7.90–7.98 (m, 1H); ^{13}C NMR (300 MHz, CD_3OD): $\delta = 164.0$ (d, $J_{C-F} = 256.0$ Hz), 139.8, 139.7 (d, $J_{C-F} = 46.0$ Hz), 135.1, 135.0, 132.4, 132.3 (d, $J_{C-F} = 9.6$ Hz), 128.9 (d, $J_{C-F} = 11.4$ Hz), 128.5, 127.80 (d, $J_{C-F} = 28.7$ Hz), 126.9, 124.4, 110.9 (d, $J_{C-F} = 24.0$ Hz); Exact mass (ESI-MS+) calcd for [$C_{13}H_9NF$]: 198.0713, found: 198.0713; Anal. calcd [$C_{13}H_9BrFN.H_2O$]: C 52.73, H 3.74, N 4.73, found: C 52.72, H 3.72, N 4.73.

2.2.3. 8,10-Difluoro-acridizinium bromide (1c)

Obtained in 32% yield as dark yellow needles, m.p. 263–265 °C (dec). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 10.37$ (s, 1H), 9.35 (s, 1H), 8.34 (d, $J = 6.7$ Hz, 1H), 8.67 (d, $J = 8.9$ Hz, 1H), 8.17 (ddd, $J = 8.4$, 7.1, 0.8 Hz, 1H), 8.04 (t, $J = 7.7$ Hz, 1H), 7.80 (ddd, $J = 9.8$, 9.2, 2.3 Hz, 1H); ^{13}C NMR (300 MHz, CD_3OD): $\delta = 162.9$ (dd, $J_{C-F} = 396.7$, 12.3 Hz), 160.4 (dd, $J_{C-F} = 402.9$, 12.4 Hz), 140.1 (bs), 139.6 (bs), 135.5, 133.3, 128.9, 128.4 (dd, $J_{C-F} = 12.7$, 4.4 Hz), 126.8, 125.2, 121.2 (dd, $J_{C-F} = 3.6$, 2.1 Hz), 111.3 (dd, $J_{C-F} = 32.9$, 23.2 Hz), 107.7 (dd, $J_{C-F} = 24.0$, 5.2 Hz); Exact mass (ESI-MS+) calcd for [$C_{13}H_8NF_2$]: 216.0619, found: 216.0618; Anal. calcd for [$C_{13}H_8F_2NBr.H_2O$]: C 49.71, H 3.21, N 4.46, found: C 49.70, H 3.27, N 4.40.

2.2.4. 8-Chloro-acridizinium bromide (1d)

Obtained in 56% yield as yellow crystals, m.p. 225–227 °C (dec). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 10.26$ (s, 1H), 9.28 (d, $J = 7.0$ Hz, 1H), 9.18 (s, 1H), 8.56 (d, $J = 8.9$ Hz, 1H), 8.51 (s, 1H), 8.38 (d, $J = 9.2$ Hz, 1H), 8.10 (m, 1H), 8.03 (dd, $J = 1.9$ Hz, 1H), 7.96 (t, $J = 6.8$ Hz, 1H); ^{13}C NMR (300 MHz, CD_3OD): $\delta = 138.9$, 138.8, 137.1, 135.6, 134.6, 134.3, 134.2, 131.6, 129.3, 127.2, 127.2, 126.0, 125.6, 123.2; Exact mass (ESI-MS+) calcd for [$C_{13}H_9NCl$]: 214.0418, found: 214.0419. Anal. calcd [$C_{13}H_9BrClN.H_2O$]: C 49.95, H 3.55, N 4.48, found: C 49.89, H 3.47, N 4.45.

2.2.5. 8-Methoxy-acridizinium chloride (1e) [16]

Obtained in 83% yield as yellow crystals, m.p. 204–206 °C (dec). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 10.02$ (s, 1H), 9.17 (d, $J = 7.0$ Hz, 1H), 9.01 (s, 1H), 8.45 (d, $J = 8.8$ Hz, 1H), 8.26 (d, $J = 9.3$ Hz, 1H), 7.97 (m, 1H), 7.86 (m, 1H) (dd, $J = 9.3$, 2.4, 1H), 7.67 (m, 1H), 4.08 (s, 3H); ^{13}C NMR (300 MHz, CD_3OD): $\delta = 162.5$, 138.0, 137. 5, 134.7, 134.1, 131.3 (2), 130.0 (2), 123.0, 128.3, 125.9, 123.9, 103.5, 57.0; Exact mass (ESI-MS+) calcd for [$C_{14}H_{12}NO$]: 210.0913, found: 10.0922.

2.3. Quantum yield determination of dyes 1a–e

Quantum yields (φ) were calculated using quinine sulfate in 1 N H_2SO_4 ($\varphi = 0.55$, excitation wavelength $\lambda_{ex} = 365$ nm) using previously published procedures [17]. The optical density of the solution at the excitation wavelength is typically <0.1 to avoid inner filter effects.

2.4. Stern–Volmer analysis

Stern–Volmer constants (K_{sv}) were determined in water at 25 °C, pH 7, with a fluorophore concentration of 300 nM. Stern–Volmer constants were calculated using data collected in the halide concentration range 0.002–0.050 M using the Microsoft Excel least squares linear regression analysis program, where regression coefficients were typically unity, *i.e.* $R^2 \geq 0.99$.

2.5. Fluorescence lifetimes

Fluorescence decay curves were measured by the time-correlated single photon counting (TCSPC) technique [18]. The excitation monochromator was set to 370 nm and the emission monochromator was set to 440 nm. Data were collected with the instrument's built-in multichannel analyzer set at a resolution of 0.1135 ns/channel. Fluorescence decay measurements were recorded in the maximum channel until 10,000 counts. Solutions were placed in a quartz tube and the instrumental response function was obtained using a silica scattering solution (Ludox). Fluorescence decays were fit by convoluting a single exponential decay with the instrumental response function using a quadrature routine, and fitting the convoluted model to the experimental data. All of the reported fits had correlation coefficients $R^2 \geq 0.99$.

3. Results

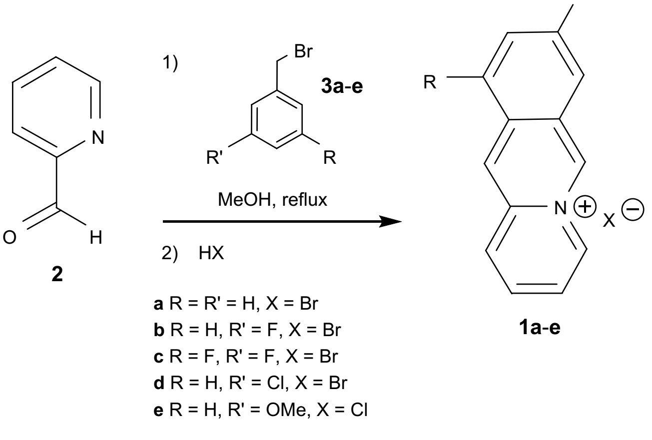
The acridizinium salts **1a**–**e** were synthesized according to an adaptation of the cyclodehydration method of Bradsher and Parham (Scheme 1) [15]. Pyridine-2-carboxyaldehyde (**2**) was quarternized *in situ* with benzyl bromides **3a**–**e**, yielding the corresponding crude pyridinium salts. Treatment with refluxing hydrobromic acid gave the acridizinium salts **1a**–**d** in moderate to good yields (32–75%). In the synthesis of **1e**, the use of hydrobromic acid led to hydrolysis of the methoxy-group. By using milder conditions, employing hydrochloric acid at 50 °C for 5 h, hydrolysis was prevented and **1e** could be obtained in 83% yield.

Absorption and fluorescence spectra of all compounds studied are presented in Fig. 2, and calculated molar absorption coefficients, absorption and emission maxima, and fluorescence quantum yields are presented in Table 1. The substituted acridiziniums displayed the expected bathochromic shift in comparison to the native fluorophore **1a**. As previously reported for this class of compounds [19], for both the native (**1a**) and halide substituted (**1b**–**d**) species display a distinct pattern of three absorption maxima in the range of 350–407 nm. However, for the 8-methoxy substituted species (**1e**) this pattern was much less pronounced with a major absorption maximum at 355 nm and weaker subsequent maxima observed between 320 and 420 nm. Fluorescence emission maxima of compounds **1a**–**e** are in the range from 404 to 455 nm with 8-methoxy substitution (**1e**) showing the largest stokes shift ($\Delta\lambda = 51$ nm). Excitation spectra of **1a**–**e** (not shown) indicate that all three absorption maxima contribute to the fluorescence at the maximum emission wavelength. Fluorescence quantum yields of the compounds studied are moderate ranging from 0.20 to 0.34.

The Stern–Volmer constants (K_{sv}, Eq. (1)) for compounds **1a**–**e** were measured to evaluate the sensitivity of the fluorophores to chloride, bromide and iodide [20,21].

$$I_0/I = \tau^0/\tau = 1 + kq\tau^0[\mathrm{Q}] = 1 + K_{sv}[\mathrm{Q}] \quad (1)$$

I_0 and I are the fluorescence intensities in the absence and presence of Q, respectively. By plotting I_0/I as a function of [Q], the value of $k_q\tau^0$ (the Stern–Volmer constant K_{sv}) can be obtained in which k_q describes the bimolecular collisional deactivation of electronic energy and τ^0 is the natural fluorescence lifetime. If quenching occurs only by a dynamic mechanism and τ^0 fits to a mono-exponential decay, then the ratio τ^0/τ will be equal to $1 + K_{sv}$. The magnitude of K_{sv} represents the sensitivity of the fluorophore towards the halide and, therefore, the concentration range that is detectable.



Scheme 1. Synthesis of acridizinium salts **1a**–**e** analogs.

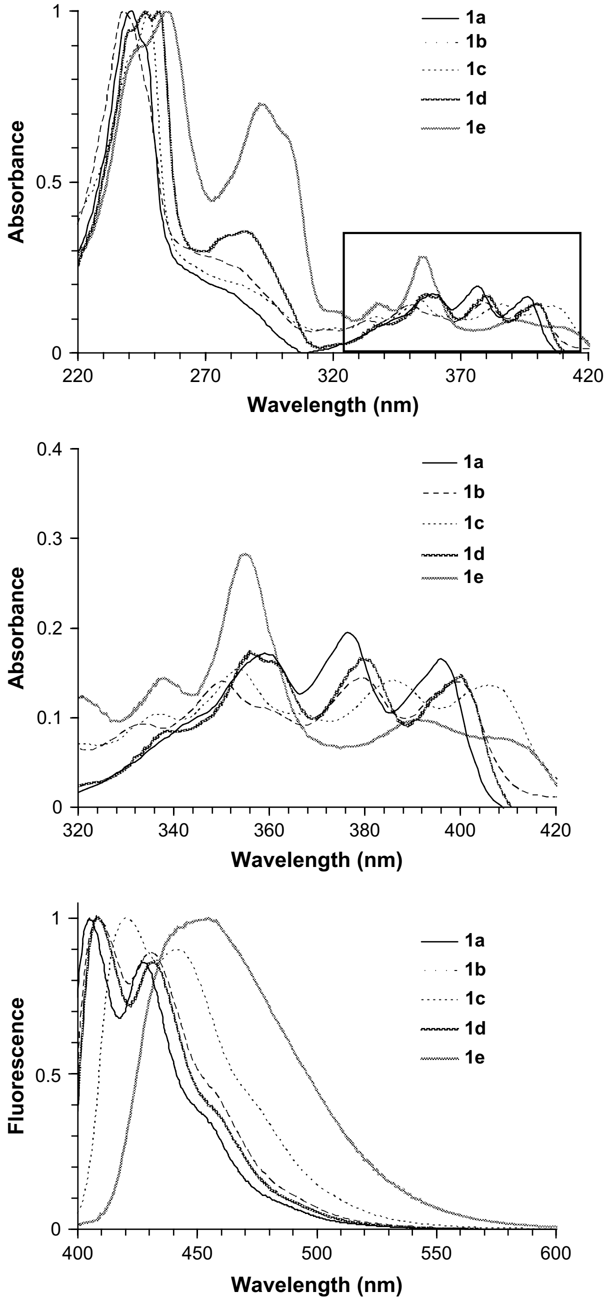


Fig. 2. Absorption spectra (upper two panels) and emission spectra at maximum absorption (lower panel) for acridizinium salts in milliQ grade water (pH 7, 25 °C).

Surprisingly, none of the studied compounds showed significant sensitivity towards chloride with $K_{sv}^{\mathrm{Cl}^-} < 1\ \mathrm{mol}^{-1}\ \mathrm{dm}^3$. Values for

Table 1
Absorption and emission maxima λ_{abs} and λ_{em}, molar absorption coefficient ε, and quantum yields φ in water.

	λ_{abs} (nm) [ε (dm^3 mol^{-1} cm^{-1})]			λ_{em} (nm)	φ^a
1a	359 [7900]	377 [8900]	396 [7600]	404	0.33 ± 0.05
1b	350 [6500]	381 [6700]	400 [6500]	408	0.34 ± 0.05
1c	354 [8400]	386 [7700]	407 [7400]	420	0.30 ± 0.05
1d	356 [5400]	380 [5200]	400 [4600]	408	0.20 ± 0.05
1e	355 [6700]	391 [2300]	406 [1900]	455	0.20 ± 0.05

[a] Mean of three separate determinations.

$K_{sv}^{Br^-}$ and $K_{sv}^{I^-}$ were measured and are listed in Table 2. All of the fluorophores gave linear Stern–Volmer relationships over the concentration range of quencher investigated, the plots of bromide quenching for the acridiziniums are shown in Fig. 3. Unsubstituted acridizinium salt **1a** showed similar sensitivity towards bromide quenching to 8-fluoro substituted **1b** and 8,10-difluoro substituted **1c**, whereas 8-chloro substituted **1d** was less sensitive towards quenching. Also the 8-methoxy substituted compound **1e** suffered from a lower sensitivity towards bromide quenching. In order to obtain a better understanding of the origin of these values, we measured both the half-wave potentials and the fluorescence decay profiles of the fluorophores **1a**–**e**. The data obtained from the fluorescence decay measurements, employing an excitation wavelength $\lambda_{ex} = 370$ nm and emission wavelength $\lambda_{em} = 440$ nm, fit well to a single exponential decay for all of the compounds investigated and gave the fluorescence lifetimes shown in Table 2. The constant k_q, which describes the bimolecular collisional deactivation of electronic energy, was calculated from the Stern–Volmer constant and the fluorescence lifetime. From this data, it becomes apparent that the reduced sensitivity of 8-chloro substituted **1d** can be explained by a shorter fluorescence lifetime compared to the other halide substituted acridizinium salts.

The half-wave reduction potentials were measured for the acridiziniums (**1a**–**e**) in aqueous solution employing potassium nitrate as a supporting electrolyte. As observed previously, the reduction of acridiziniums is not reversible, thus the half-wave potentials only serve as an indication of the reduction potential of the compounds [24]. The reduction potentials of the halide substituted derivatives (**1b**–**d**) are less negative than the native acridizinium (**1a**), and the methoxy (**1e**) derivative is similar to the native acridizinium.

4. Discussion

The acridizinium fluorophores can be readily synthesized by the aromatic cyclodehydration of commercially available pyridine-2-carboxaldehyde and the corresponding benzyl bromides. The ease of access to these fluorophores makes them promising candidates for the development of more complex sensors based on halide exchange mechanisms [3].

The spectroscopic analysis shows the acridiziniums have significantly improved properties over the other common halide sensitive fluorophores, the quinolium and acridinium salts. The acridiziniums (**1a**–**e**) absorb at longer wavelengths than the quinolinium fluorophores ($\lambda_{abs} = 320$–350 nm, $\varepsilon = 3450$ L mol^{-1} cm^{-1}) and **1a**–**d** have approximately two-fold higher extinction coefficients than the acridiniums at wavelengths above 400 nM [4]. The quantum yields of the acridiziniums was lower than that of the quinoliniums or acridiniums but in sensing applications this is partially compensated for by the increased extinction coefficients.

The lack of chloride quenching by compounds **1a**–**e** was unexpected as significant quenching had been observed for 9-chloroacridizinium previously in methanol [14]. Detailed studies of the halide quenching mechanism for a series of quinolinium derivatives has suggested that halide quenching is mediated by a non-emissive excited state charge transfer complex [22]. Access by the quinolinium fluorophores to this quenching manifold correlates with the increasing reduction potential (more positive) and the increased singlet state excitation energy of the fluorophores, as well as the oxidation potential of the quenching ion [22]. The quinolinium salts have reduction potentials of −0.9 to −0.7 V and excitation wavelengths of between 320–350 nm. The reduction potential of the acridizinium salts (see Table 2) [23,24] can only be estimated from the reduction half-wave potentials, due to the irreversibility of the reduction. From these estimations, the reduction potentials are expected to be more positive than those of the quinoliniums (−0.8 to −0.6) and thus favoring quenching by chloride. The more positive reduction potentials are, however, offset by the longer wavelengths of excitation of the acridiziniums which lead to decreased singlet state energies in comparison to the quinoliniums. Given that quenching with chloride was observed for

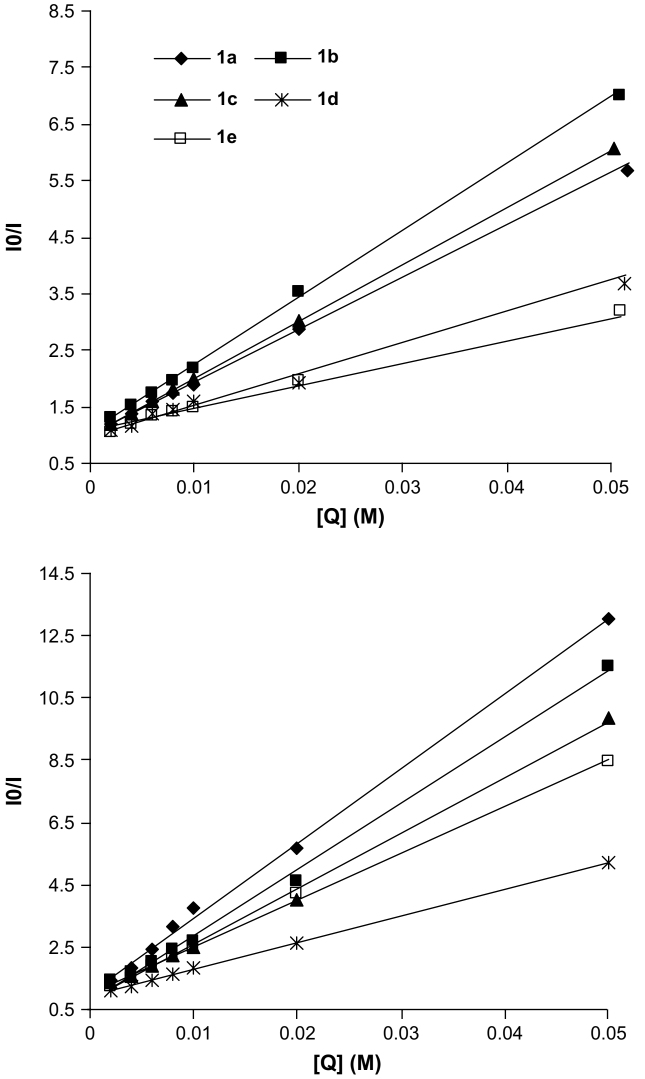


Fig. 3. Stern–Volmer plot of **1a**–**e** for bromide (upper panel) and iodide (lower panel) quenching.

Table 2
Half-wave potentials ($E_{1/2}$), normal fluorescence lifetime (τ^0), and Stern–Volmer constants (K_{sv}) for quenching with KBr and KI measured in doubly distilled deinoised H_2O (pH 7, 25 °C) together with the calculated bimolecular quenching constant (k_q) for **1a**–**e**.

Dye	$-E_{1/2}$	τ^0 (ns)[a]	λ_{ex} (nm)	λ_{em} (nm)	K_{sv} (mol^{-1} dm^3)		k_q (mol^{-1} ns^{-1} dm^3)[b]	
					Br^-	I^-	Br^-	I^-
1a	0.89[c]	6.0	396	404	90	250	16	42
1b	0.73	6.2	381	408	120	180	20	29
1c	0.65	5.4	407	420	100	150	19	28
1d	0.66	3.6	356	408	60	90	15	26
1e	0.89[c]	7.5	355	455	40	160	5.5	21

[a] The normal fluorescence lifetime (τ^0) was measured with excitation wavelength $\lambda_{ex} = 370$ nm and emission wavelength $\lambda_{em} = 440$ nm.
[b] Calculated from τ^0 and K_{sv}.
[c] In accordance with literature, see Ref. [24].

9-chloro-acridizinium in methanol, and that this compound has a similar half-wave reduction potential (−0.8 V) and excitation wavelength (396 nm) to the acridiziniums investigated here, it was hypothesized that the lack of quenching may be due to the solvation of the chloride ions in aqueous solution. Measurements of the quenching of acrdizinium bromide (**1a**) with chloride in acetonitrile confirms this hypothesis as significant quenching is observed ($K_{sv} = 320\ \mathrm{mol}^{-1}\ \mathrm{L}^{-1}$).

The sensitivity to bromide and iodide of the acridiziniums in aqueous solution is substantial. A Stern–Volmer coefficient of 100 ($\mathrm{mol}^{-1}\ \mathrm{L}^{-1}$) leads to approximately 50% quenching of the fluorophore in a 10 mM solution of the given quencher. Thus, all of the acridiziums investigated would be useful for measuring bromide or iodide concentrations in the high micromolar to mid millimolar range. Despite the lack of chloride sensitivity of the acridiziniums, these fluorophores offer several benefits when used to quantify bromide or iodide ions over the quinolinium or acridinium fluorophores. In comparison with the quinolinium fluorophores, the longer wavelengths of excitation (>400 nm vs. 350 nm) significantly reduce the experimental challenges when using these fluorophores in environments with autofluorescence at low excitation wavelengths such as in biological solutions. In comparison to the acridinium fluorophores, known to decompose in minutes in physiological solutions, the acridizinium fluorophores were significantly more stable under physiological conditions. Solutions of compounds (**1a**–**e**) were routinely made up days in advance of experiments without significant losses in fluorescence [4]. The increased stability of the acridiziums is due to the fact that they are not susceptible to the same nucleophilic addition mechanism as the acridiniums at position 9.

The introduction of fluorine substitutions to other fluorophores have also shown improved properties over the native fluorophore skeleton [25]. In our case, comparison of the properties of differentially subsitituted acridiziniums indicate that the 8,10-difluoro substitution pattern, acridizinium **1c**, leads to the best combination of properties having the longest wavelength of excitation, a high quantum yield and good sensitivity to both bromide and iodide ions.

5. Conclusions

Acridizinium fluorophores provide substantially improved properties over quinolinium and acridinium fluorophores for applications involving the sensing of bromide and iodide in physiological solutions. The 8,10-difluoro substituted acridizinium **1c**, provides the best combination of halide sensitivity and photophysical properties.

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