

Syntheses of Fluorescent Dyes. XIV.
Standards for Fluorescence Measurements in the Near Neutral
pH-Range (1)

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Highly fluorescent cationic indicators have been obtained by reaction of heterocyclic nitrogen bases with 1,3-propane sultone. Unlike the bases themselves, the fluorescence intensity of the cationic salts is practically *pH*-independent in the neutral *pH*-range, which makes them useful reference substances in measuring fluorescence intensities in physiological studies. Furthermore, they are sufficiently water soluble, the fluorescence maxima covering the wavelength range from 440 to 530 nm. 2-(3-Sulfonatopropyl)harmanium, (**5**) appears to be the most useful compound for intra- and extracellular liquids, since its fluorescence is not quenched by chloride ion.

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Fluorescence quantum yields of indicators can be determined either absolutely, or relatively to a standard substance. For aqueous solutions the critical review of Demas and Crosby (2) indicates only two systems, namely quinine bisulfate in 1 *N* sulfuric acid ($\phi_f = 0.546$) and fluorescein in 0.1 *N* sodium hydroxide ($\phi_f = 0.90$), whose fluorescence yields were probably reliable to within 5%. However, both of these indicators are not suitable for measurements in the near neutral *pH*-range, since under these conditions the fluorescence intensities of the two standards are highly *pH*-dependent (3).

Consequently we were looking for fluorescence standards having the following properties: a) *pH*-Independence of the emission intensity at near neutral *pH*'s; b) High fluorescence quantum yields; c) Large Stokes shift; d) Good water solubility.

We perceived, that quarternisation of heterocyclic bases would have the same beneficial effect on fluorescence as

protonation has. Protonation, however, is a *pH*-reversible process, whilst quarternisation is irreversible, thus making the fluorescence of the compounds invariably with *pH*, at least in the *pH* range from 0 to 9.

The reagent of choice for quarternisation appears to be the readily available 1,3-propane sultone. The reaction products with nitrogen bases would require no counterion, since the resulting molecule as a whole is uncharged. 6-Methoxyquinoline, acridine, phenazine, acridine orange and harman (1-methyl-9*H*-pyrido[3,4-*b*]indole) were submitted to quarternisation. Reaction was simply accomplished by melting together propane sultone and the base for 30 to 120 minutes at 100 to 130°. The structures of the resulting compounds **1**, **2**, **3**, **4**, **5** are given in the formula Scheme.

All compounds **1** to **5** are highly fluorescent, covering the emission wavelength range from 400 to 560 nm (Table 1). The fluorescence intensities are *pH*-independent in

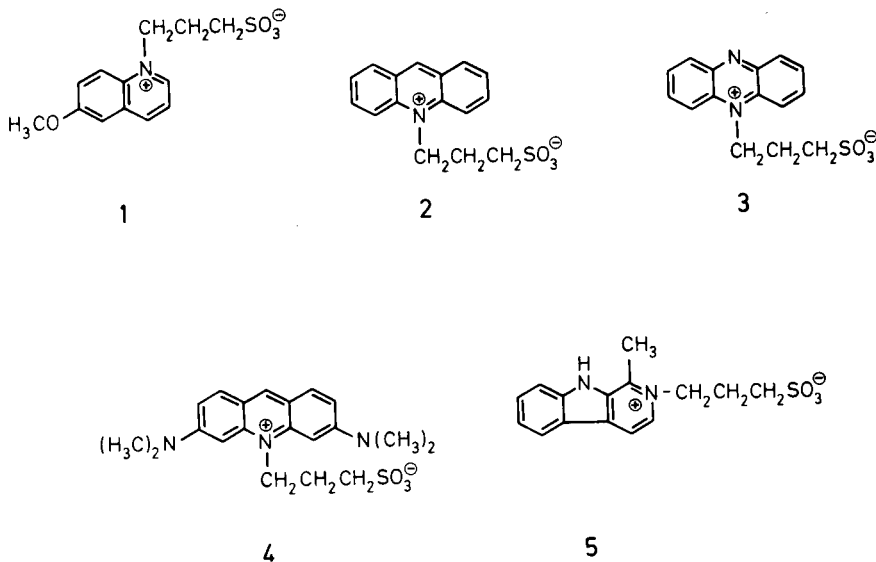


Table 1

Absorption and Fluorescence Data, Stokes Shifts, Fluorescence Quantum Yields and Solubilities of Compounds **1** and **5**

Compound	λ abs max (in nm)	$\log \epsilon$ ($M^{-1} \text{ cm}^{-1}$)	λ flu max (in nm) (a)	ϕ_f (b) (d)	water solubility
1	344	3.63	450	0.53	very good
	317	3.81			
2	415	3.71	488	1.00 (e)	good
	358	4.43			
3	434	3.50	529	(c)	very good
	387	4.39			
4	494	4.12	525	—	poor
5	367	3.80	441	0.80	fair

(a) Uncorrected; (b) Quantum yields relative to quinine sulfate; (c) Too unstable for sufficiently accurate determination; (d) Excitation wavelength range 340 to 345 nm; (e) Relative to *N*-methylacridinium chloride [$\phi_f = 1.01$: G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **53**, 646 (1957)].

the neutral pH-range. Stokes shifts are large, ranging from 73 to 106 nm, except for **4** (31 nm). Also, **4** lacks good water solubility, whilst **1**, **2** and **3** are readily soluble, **5** dissolves sufficiently. Solutions of compounds **1**, **2** and **5** are fairly stable and can be stored. There are, however, colour changes to be observed at higher pH's: Yellow solutions of the phenazinium salt **3** turn purple at pH's higher than 9. Similarly, the green fluorescence of acridinium salt **2** turns to violet in alkaline solutions ("pK_a" 10.01), probably as a result of hydroxide ion addition in 9-position. Consequently, compounds **1**, **2** and **5** appear to be the most useful fluorescence standards. Table 1 compiles the relevant properties of the indicators.

Like the quinine cation, the fluorescence of compounds **1** and **2** is quenched by halide ions, which, in studying cellular liquids may be a severe obstacle and a potential source of error. We have found, that the harmanium salt **5** is not quenched by up to 1 M concentrations of chloride and thus appears to be a superior indicator, which we can recommend.

EXPERIMENTAL

Melting points are uncorrected, ir spectra were obtained using a Perkin Elmer 421, and ¹H nmr spectra on a Varian A 60A. Chemical shifts and coupling constants are given in δ -units and in Hertz, respectively. Absorption spectra were run on a Hitachi 110 spectrophotometer, fluorescence spectra at room temperature on an Aminco SPF 500. Ten mg samples of these indicators may be ordered from the authors.

6-Methoxy-1-(3-sulfonatopropyl)quinolinium (**1**).

6-Methoxyquinoline (0.6 g, 3.77 mmoles) was carefully mixed with 1.0 g (8.17 mmoles) 1,3-propanesultone and heated on a water bath under exclusion of moisture for half an hour. The liquid mixture solidified to white crystals, which were sparingly soluble in alcohol and very soluble in water. Recrystallisation from 30 ml of water-ethanol (1:1, v/v) gave a 90% yield (0.95 g) of **1**, mp 299° dec; ir (potassium bromide): 1625, 1595, 1530, 1395, 1282, 1192, 1034 and 850 cm^{-1} ; ¹H nmr (deuterium oxide): 2.73 (m, 2H), 3.30 (t, 2H), 4.20 (s, 3H), 5.36 (t, 2H), 7.7-9.4 (m, 6H).

Anal. Calcd. for C₁₃H₁₅NO₃S (281.34): C, 55.50; H, 5.37; N, 4.98; S, 11.40. Found: C, 55.42; H, 5.34; N, 4.88; S, 11.20 (4).

10-(3-Sulfonatopropyl)acridinium (**2**).

Acridine (1.0 g, 5.58 mmoles) and 1.5 g (12.25 mmoles) of 1,3-propanesultone were heated together to 110 to 120° under exclusion of moisture for two hours. Still warm, the brown mass was treated with 5 ml of dimethylformamide (DMF) and was allowed to stand for 2 hours. The resulting crystals were removed by suction and recrystallised from 70 ml of alcohol (charcoal), yield, 70% (1.18 g) of greenish yellow needles, decomposing at 264-266°; ir (potassium bromide): 1618, 1390, 1225, 1200, 1162, 1035, 775, 757 and 730 cm^{-1} ; ¹H nmr (DMSO-d₆): 1.9 (m, 2H), 2.90 (t, 2H), 5.73 (t, 2H), 7.9-9.2 (m, 8H), 10.2 (s, 1H).

Anal. Calcd. for C₁₆H₁₅NO₃S (301.37): C, 63.77; H, 5.01; N, 4.65; S, 10.64. Found: C, 63.63; H, 5.10; N, 4.57; S, 10.55 (4).

5-(3-Sulfonatopropyl)phenazinium (**3**).

Phenazine (1.0 g, 5.55 mmoles) was fused with 2.0 g (16.33 mmoles) of 1,3-propanesultone at 130° and kept at this temperature for one hour. The mixture was treated with 5 ml of DMF and kept at 5° for several hours. The resulting precipitate was collected by suction, washed with acetone and crystallised from about 150 ml of ethanol-water (9:1). Long boiling has to be avoided in order to prevent a green decolouration of the yellow solution. Dilute aqueous solutions are of limited stability, addition of alkali causes a colour change to purple, yield 40 to 60% (0.67-1.00 g) of reddish brown crystals, dec range 224-230°; ir (potassium bromide): 1600, 1362, 1200, 1159, 1030, 769 and 725 cm^{-1} ; ¹H nmr (deuterium oxide): 2.9 (m, 2H), 3.55 (t, 2H), 5.85 (t, 2H), 8.3-9.3 (m, 8H).

Anal. Calcd. for C₁₅H₁₄N₂O₃S (302.65): C, 59.53; H, 4.66; N, 9.26; S, 10.59. Found: C, 59.67; H, 4.88; N, 9.16; S, 10.44.

3,6-(bis-Dimethylamino)-10-(3-sulfonatopropyl)acridinium (**4**).

The free base of acridine orange (1.0 g, 3.77 mmoles), prepared from the commercial hydrochloride by addition of dilute sodium hydroxide solution and recrystallisation from ligroin) was mixed with 1.0 g (8.17 mmoles) of 1,3-propanesultone and heated to 110-120° for one hour. Treatment of the cold melt with 5 ml of DMF causes crystallisation. The precipitate was crystallised from 40 ml of DMF to give a 60% yield (0.88 g) of **4**, red crystals, dec 334°; ir (potassium bromide): 1600, 1500, 1363, 1205, 1195, 1160, 1037 and 805 cm^{-1} .

Anal. Calcd. for C₂₀H₂₅N₃O₃S (387.49): C, 61.99; H, 6.50; N, 10.85; S, 8.27. Found: C, 62.22; H, 6.53; N, 10.89; S, 8.30 (4).

1-Methyl-2(sulfonatopropyl)-2H-pyridio[3,4-b]indolium (**5**).

Harman (0.3 g, 1.65 mmoles) and 0.3 g (3.45 mmoles) of 1,3-propanesultone were allowed to react as described for **3**.

Recrystallisation from methanol afforded 0.35 g (71%) of colourless crystals of dec 315°; ir (potassium bromide): 1645, 1220, 1185, 1160, 1140, 770, 760 and 728 cm^{-1} .

Anal. Calcd. for $\text{C}_{15}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$ (304.37): C, 59.18; H, 5.30; N, 9.21; S, 10.53. Found: C, 59.45; H, 5.11; N, 9.05; S, 10.39 (4).

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REFERENCES AND NOTES

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- (2) J. N. Demas and G. A. Crosby, *J. Phys. Chem.*, **75**, 991 (1971).
- (3) G. G. Guilbault, "Practical Fluorescence-Theory, Methods, and Techniques", Marcel Dekker, New York, 1973, p 599.
- (4) In order to get correct elemental analyses of the water-free compounds they have to be dried for several hours at 130° under vacuum over phosphorus pentoxide.