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Intracellular imaging of organelles with new water-soluble benzophenoxazine dyes†

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Five new water-soluble derivatives of Nile Red **1–5** were prepared. These benzophenoxazine dyes fluoresce between 640 and 667 nm with quantum yields of 0.17–0.33 in pH 7.4 phosphate buffer, and at slightly shorter wavelengths and higher quantum yields in EtOH. Two dyes, **3** and **4** permeated into Clone 9 cells and selectively stained mitochondria and golgi, respectively.

Introduction

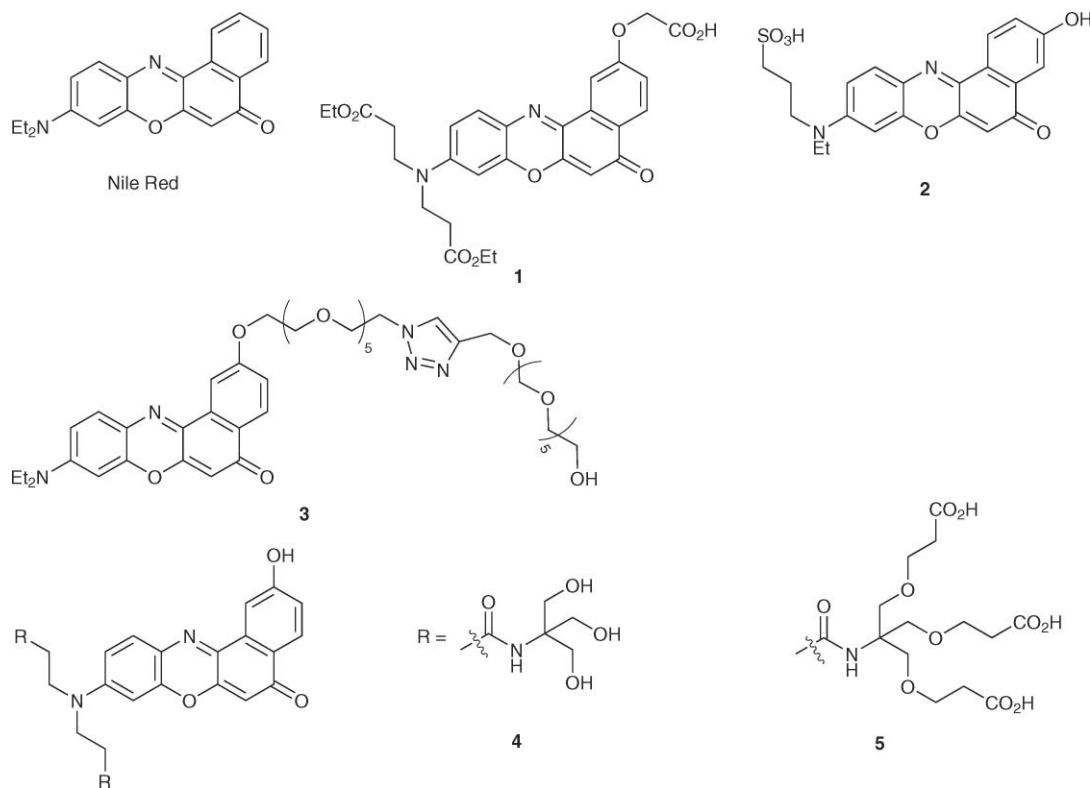
Nile Red is a solvatochromic benzophenoxazine dye. In non-polar solvents it fluoresces at significantly shorter wavelengths, and with much higher quantum yields, than in polar media. Consequently, Nile Red is used as a lipophilic stain for detection of intracellular lipid droplets.^{1,2}

Applications of Nile Red in cellular imaging³ are limited because it is insoluble and non-fluorescent in aqueous media, probably due to aggregation. Both these limitations could be overcome by introducing water-soluble groups onto the benzophenoxazine scaffold, but there are very few reports of water-soluble benzophenoxazine dyes in the literature.^{4–6} Herein we report the new water-soluble Nile Red derivatives **1–5**, and their photophysical properties in aqueous media. They fluoresce strongly in aqueous media at wavelengths (>640 nm) that can be easily differentiated from cellular autofluorescence. Consequently, the ability of these dyes to permeate into cells was examined, and two, **3** and **4**, are shown to be cell permeable, and to selectively localize in the mitochondria and in the golgi, respectively.

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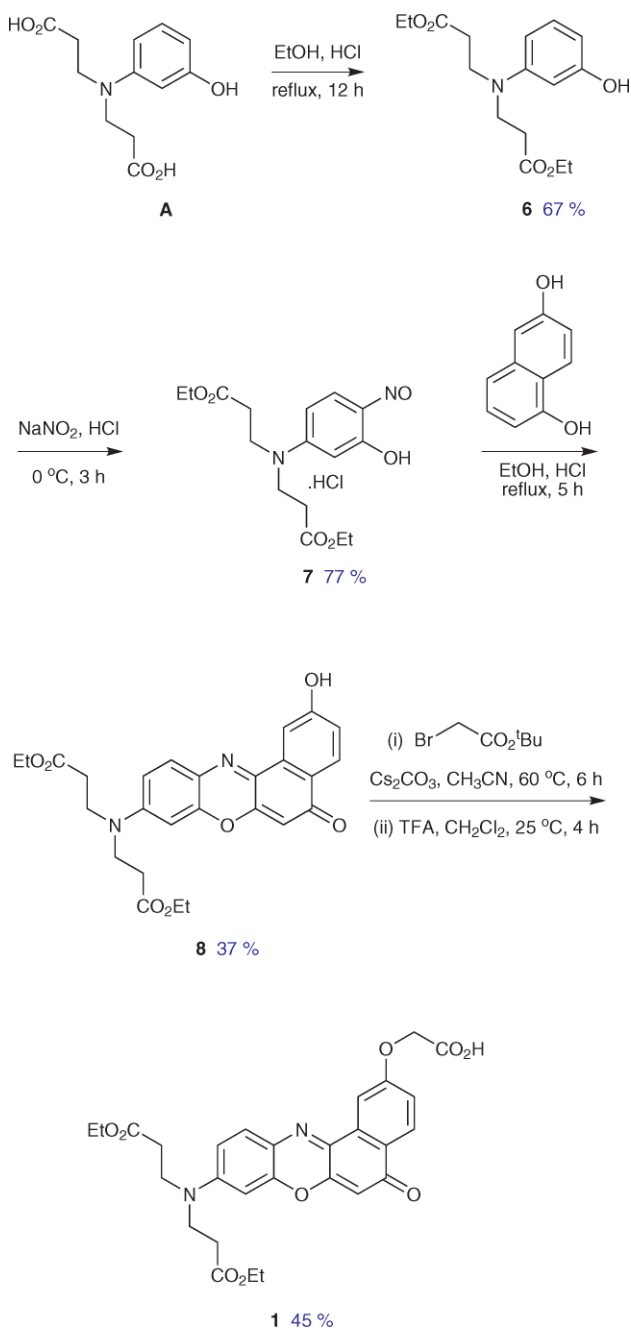
† Electronic supplementary information (ESI) available: Synthesis of dyes **1–5**; copies of ¹H, ¹³C NMR and mass spectrum. See DOI: 10.1039/b925845k



Results and discussion

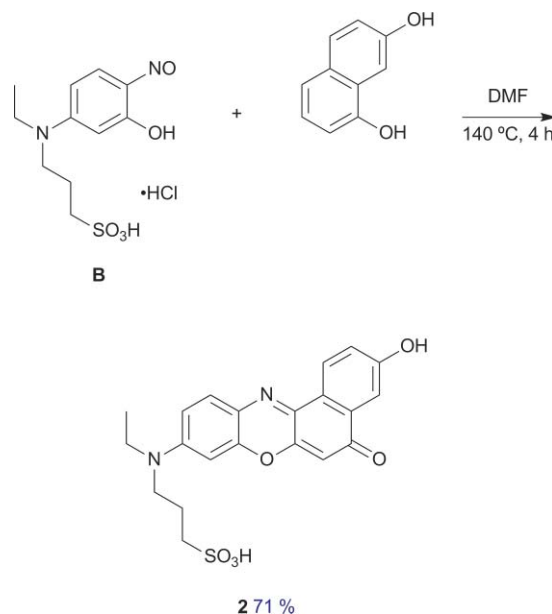
Design and synthesis

The synthetic strategies used here involved adding water solubilizing functionalities to the phenol of hydroxy Nile Red isomers, or attaching other groups to the 9-amino substituent. Synthesis of dye **1** began with the dicarboxylic acid **A** (Scheme 1).⁶ Esterification, nitrosation then condensation with 1,6-dihydroxy naphthalene afforded dye **8**. Alkylation of this with *tert*-butyl bromoacetate followed by removal of *t*-butyl group afforded **1** in 45% yield. Somewhat surprisingly,⁷ dye **1**, that has only one highly polar substituent, was sufficiently soluble in water for fluorescence studies.



Scheme 1 Synthesis of dye **1**.

Sulfonic acid **2** (Scheme 2) was prepared from nitroso compound **B**.⁸ Reaction of this compound with 1,7-dihydroxy naphthalene in DMF at 140 °C yielded **2** in 71% yield. A similar dye is reported in the literature but its photophysical properties in aqueous media were not reported.⁵ The presence of a phenolic group and a sulfonic acid group makes dye **2** highly water-soluble.



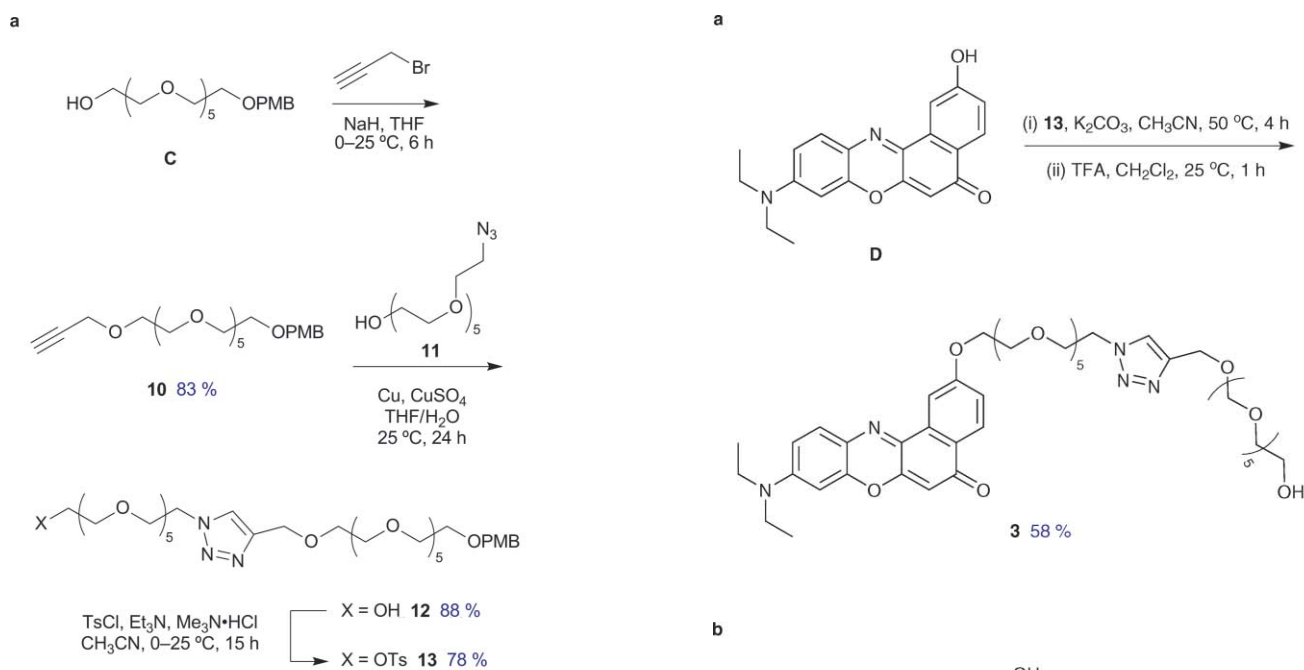
Scheme 2 Synthesis of dye **2**.

Dyes **3–5** feature the water-solubilizing groups **13–15**. The “click-derived” oligoethylene glycol fragment from linker **13** is accessible in gram quantities (Scheme 3a).⁹ Trizma base **14** is widely used as a buffer (pH 8.4) in immunohistochemical staining and is very soluble in water; it is a useful template for incorporating multiple carboxylic acid groups for improved water-solubilities and attachment to biomolecules. Thus treatment of **14** with acrylonitrile followed by acid hydrolysis yielded **15** in quantitative yield (Scheme 3b).¹⁰ It was hypothesized that the cone-like shapes of **14** and **15** tend to prevent π -stacking of dyes and reduce aggregation in aqueous media.

To prepare dye **3**, 2-hydroxy Nile Red **D**⁵ was alkylated with oligoethylene glycol linker **13** followed by removal of PMB group in TFA/ CH_2Cl_2 to afford **3** in 58% yield (Scheme 4a). Scheme 4b shows the synthesis of **4** and **5** from the water soluble Nile Red derivative **E**.⁶ Activation of the carboxylic acids then reaction with corresponding amines yielded dyes **4** and **5**. The longer reaction times required for **5** may be attributed to the steric bulk of the water-soluble group **15**. Dyes **4** and **5** were purified on medium pressure liquid chromatography using reverse phase C-18 column and $\text{CH}_3\text{CN-H}_2\text{O}$ as eluent.

Photophysical properties of dyes in aqueous and organic media

Compounds **1–5** showed good solubilities in water, pH 7.4 phosphate buffer, and EtOH. Absorbance and fluorescence spectra of the dyes in phosphate buffer pH 7.4 and in EtOH are shown in Fig. 1 and 2. Their absorbance and fluorescence maxima are red shifted by 20–30 nm in buffer compared to EtOH

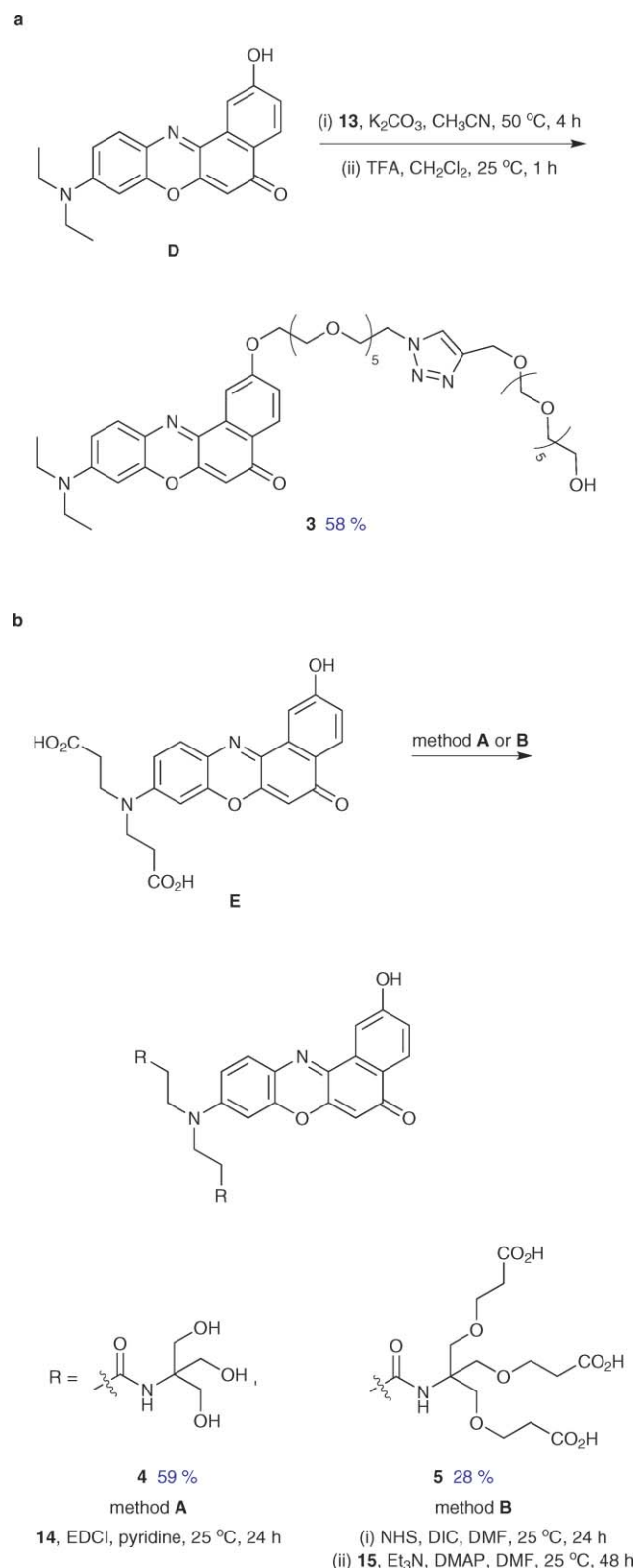


(Fig. 2). The Stokes' shifts of these dyes vary between 78–99 nm; this is an advantage when observing the fluorescence output with less interference from the light source used to excite the dye.

Table 1 and 2 show the photophysical properties of **1–5** in pH 7.4 buffer and in EtOH. Quantum yields of the dyes in EtOH varied from 0.47–0.73. The quantum yields measured for dyes **1–5** in buffer were less, but still significantly better than 2-hydroxy Nile Red dye **D** which is almost non-fluorescent in buffer. Relative to this standard, the different water-solubilizing groups have a positive influence on the solubility and quantum yield of dyes in aqueous media, albeit to different extents. Sharpness of fluorescence emissions tend to be inversely related to aggregation effects. The full width at half maxima values for dyes **1–5** in buffer are 53–65 nm; this is relatively sharp, and better than the same compounds in EtOH (Table 2).

Cellular imaging studies

Intracellular localization of the water-soluble Nile Red derivatives **1–5** was compared to 2-hydroxy Nile Red **D** using Clone 9 cells.



When the cells were incubated with **D**, specific labelling of the golgi apparatus was observed (Fig. 3a). Compound **3**, however, targeted the mitochondria (Fig. 3b) while dye **4** also localized in the golgi

Table 1 Photophysical properties of dyes 1–5 in pH 7.4 buffer

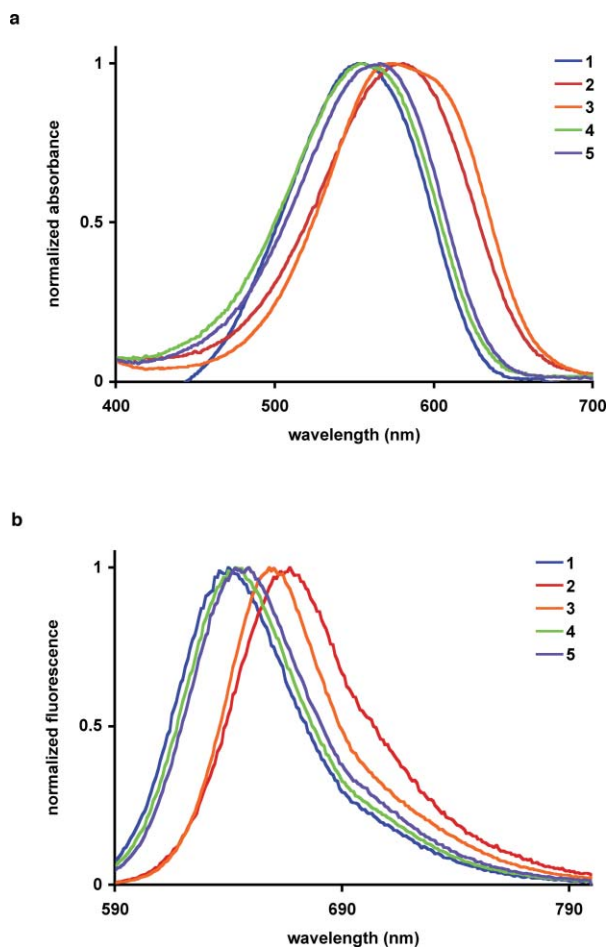
Dye	$\lambda_{\text{abs}}/\text{nm}$	$\varepsilon/\text{M}^{-1}\text{cm}^{-1}$	$\lambda_{\text{em}}/\text{nm}$	Fwhm ^a /nm	Φ
1	554	14300	640	60	0.33±0.01 ^b
2	581	15200	667	65	0.17±0.01 ^c
3	572	15250	658	53	0.26±0.02 ^b
4	554	7900	643	60	0.32±0.01 ^b
5	566	8300	654	63	0.28±0.02 ^b

^a Fwhm: full width at half maximum for the fluorescence band. Standards:^b Rhodamine 101 in EtOH (Φ :1.0),¹¹ ^c Sulforhodamine in EtOH (Φ :1.0),¹² quantum yield measurements were repeated three times and standard deviations are shown.

Table 2 Photophysical properties of dyes 1–5 in EtOH

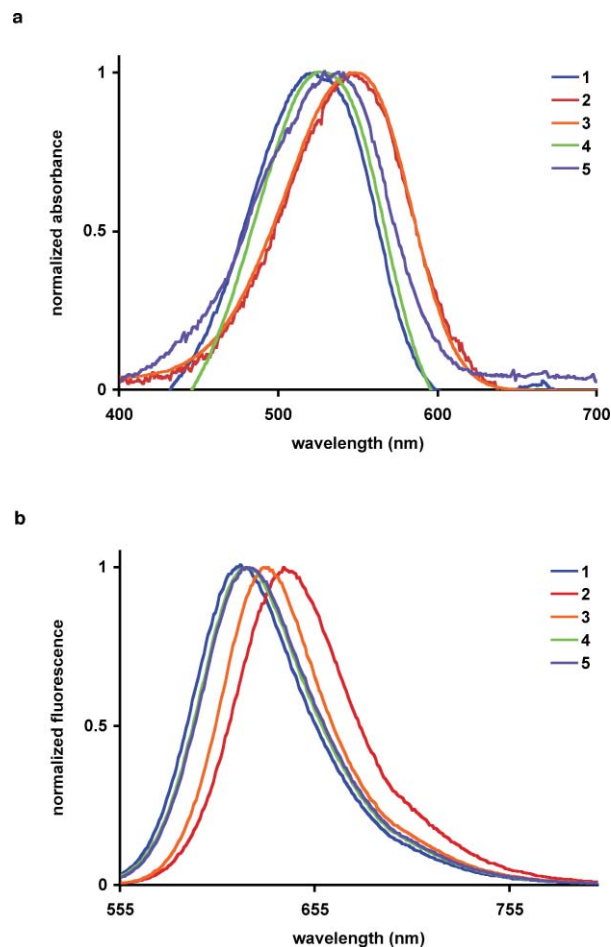
Dye	$\lambda_{\text{abs}}/\text{nm}$	$\varepsilon/\text{M}^{-1}\text{cm}^{-1}$	$\lambda_{\text{em}}/\text{nm}$	Fwhm ^a /nm	Φ^b
1	522	15400	621	65	0.73±0.03
2	545	16200	639	66	0.60±0.01
3	548	11740	631	60	0.48±0.01
4	530	9900	620	65	0.47±0.02
5	544	10200	622	67	0.63±0.01

^a Fwhm: full width at half maximum for the fluorescence band. Standard:^b Rhodamine 6G in EtOH (Φ :0.94),¹¹ quantum yield measurements were repeated three times and standard deviations are shown.

**Fig. 1** Normalized absorption (a) and fluorescence (b) spectra of dyes 1–5 in pH 7.4 buffer. Absorbance at 10^{-6} M; fluorescence at 10^{-7} M.

(Fig. 3c). Localization of **D** and **4** in the golgi was confirmed by comparing their staining pattern to the one of BODIPY TR ceramide complexed to BSA, a commercial (Invitrogen) marker for golgi (see ESI†).

The negatively charged Nile Red derivatives **1**, **2** and **5** did not enter the cells under the same conditions that were used for compounds **3** and **4** above. We have previously discovered non-covalently bound, oligo-arginine carriers like azoR₈ mediate import of proteins into lysosomes when incubated with cells at 37 °C.¹³ We hypothesized that the same carrier might also be useful for import of some negatively charged dyes. To test this,

**Fig. 2** Normalized absorption (a) and fluorescence (b) spectra of dyes 1–5 in EtOH. Absorbance at 10^{-6} M; fluorescence at 10^{-7} M.

5 was mixed with azoR₈ (1 : 3, dye : carrier) and incubated with the Clone 9 cells for 1 h at 37 °C. As expected the probe could be observed inside the cells, but it was sequestered in lysosomes (Fig. 3d) as evidenced by co-localization with LysoTracker Blue DND-22 (Invitrogen).

It is interesting that stain **3** selectively collects in the mitochondria. Mitochondria possess a net negative charge on the matrix side of the membrane, hence common markers for this organelle tend to be lipophilic fluorophores with delocalized positive charges *e.g.* Mitotracker green (a cyanine based dye) or Mitotracker red (a rhodamine dye), both from Invitrogen.

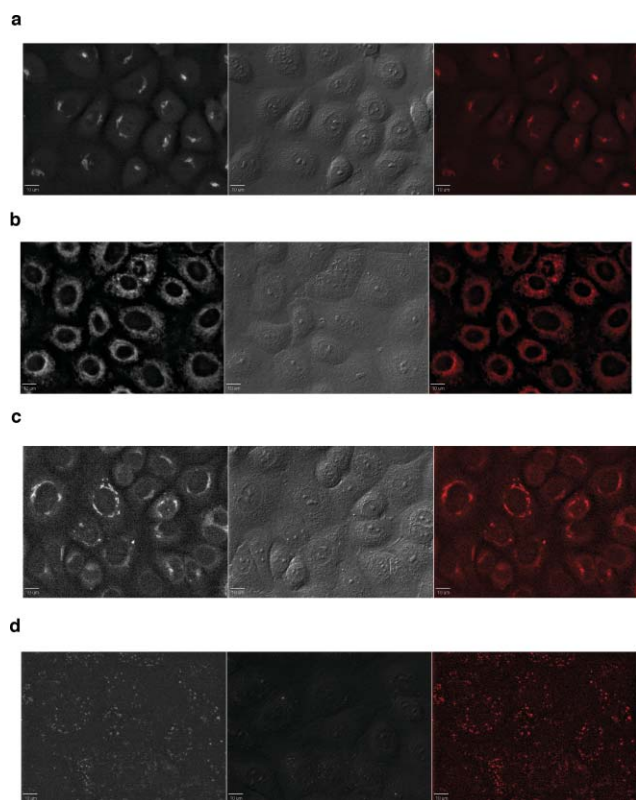


Fig. 3 Images of Clone 9 cells treated with (a) dye **3** (2 μM , 30 min, 37 $^{\circ}\text{C}$ in ACAS); (b) dye **3** (5 μM , 30 min, 37 $^{\circ}\text{C}$ in ACAS); (c) dye **4** (0.5 μM , 30 min, 37 $^{\circ}\text{C}$ in ACAS); and (d) a 1:3 dye **5**:azoR₈ (60 min, 37 $^{\circ}\text{C}$ in ACAS).

In an attempt to understand the mechanism of uptake of **3**, Clone 9 cells were co-incubated with **3** and a cyanine based dye called JC-1¹⁴ that accumulates in mitochondria. The special characteristic of JC-1 is that it fluoresces green ($\sim 529\text{ nm}$) when in normal polarized mitochondria, but red ($\sim 590\text{ nm}$) when the mitochondria are in a stressed, depolarized state. Thus JC-1 indicates mitochondrial depolarization by a decrease in the red/green fluorescence intensity ratio. Fig. 4a shows that treatment of the cells with **3** results in a decrease of the JC-1 red/green fluorescence intensity ratio indicative of decreased mitochondrial membrane potential. This response was dose-

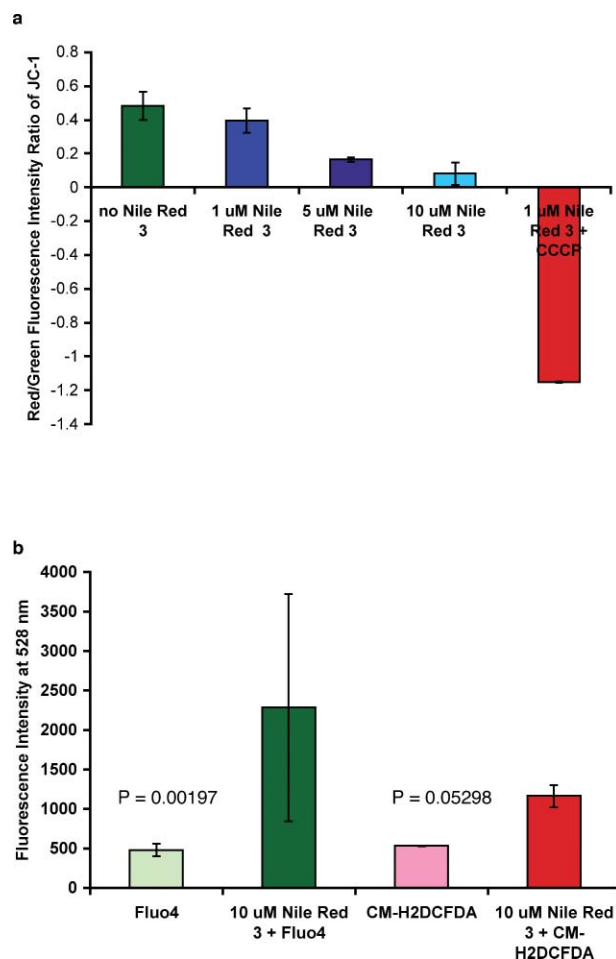
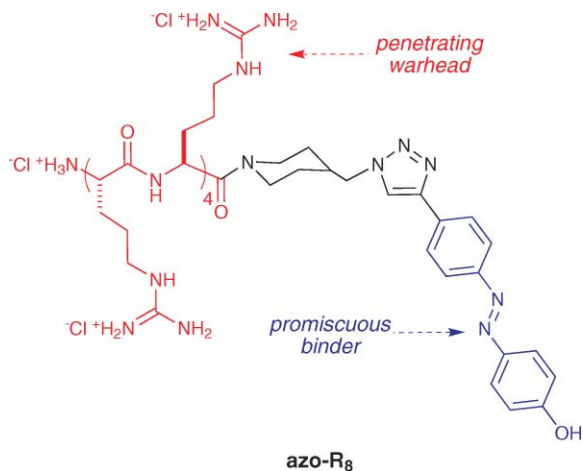
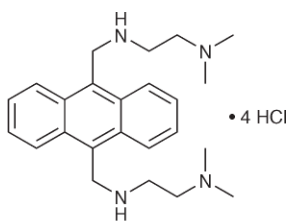


Fig. 4 a Treatment with **3** decreases the mitochondrial membrane potential but does not completely depolarize it at the indicated concentrations - Throughout, 5 $\mu\text{g mL}^{-1}$ of JC-1 was used. CCCP was used at 100 μM for 30 min at 37 $^{\circ}\text{C}$ to ensure complete depolarization of the mitochondria.; b mitochondrial depolarization is associated with an increase in the intracellular calcium concentration and increase of ROS. Fluo 4 and CM-H₂DCFDA were used at 3 μM , and 2 $\mu\text{g mL}^{-1}$, respectively. Throughout 10 μM of **3** was used.

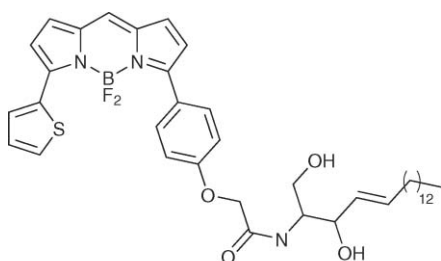


dependant with respect to **3**. CCCP is a compound that completely depolarizes mitochondria;¹⁵ this control showed that 1–10 mM concentrations of **3** depolarized the mitochondria, though not completely.

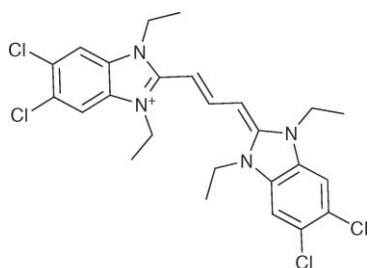
Depolarization of mitochondria tends to be associated with increased calcium ion concentrations.^{16–19} To explore this, Clone 9 cells were incubated with **3** and the calcium sensitive probe Fluo-4;²⁰ the fluorescence increase, indicative of calcium ion concentration, was greater than in a control experiment wherein **3** was omitted (Fig. 4b). Depolarization of mitochondria also tends to be associated with increased concentrations of reactive oxygen species (ROS).²¹ When the Clone 9 cells were incubated with **3** and the ROS indicator CM-H₂DCFDA then the fluorescence output also increased relative to a control where **3** was not present (Fig. 4b; see ESI for full details†).



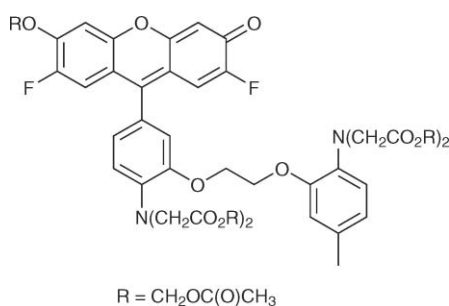
LysoTracker Blue DND-22
lysosome marker



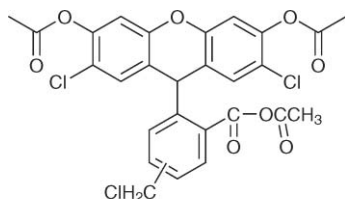
BODIPY TR ceramide
probe for golgi apparatus



JC-1
mitochondrial potential sensor



Fluo-4
calcium sensor



CM-H₂DCFDA
ROS sensor

Conclusions

Water-soluble dyes **1–5** fluoresce with good quantum yields in aqueous media, at wavelengths that are greater than those associated with cellular autofluorescence and with large Stokes' shifts. Stains **3** and **4** are cell permeable and organelle selective. Dye **3**, unlike commercially available stains for mitochondria, is *not* positively charged. This stain was shown to have some depolarizing effects on mitochondria. Stain **4** is different to most commercial stains for the golgi, which tend to be based on ceramide derivatives, *e.g.* BODIPY TR ceramide complexed to BSA. Nile Red has been known for well over 100 years, but has never been applied in intracellular imaging. Overall, these studies show that common lipophilic dyes that are modified to be water-soluble, can afford interesting new stain types for organelles in living cells.

Experimental

See the supplemental data for detailed synthesis and characterization informations of intermediate compounds, and cellular imaging studies.[†]

Synthesis of 1

A solution of **9** (100 mg, 0.17 mmol) (see ESI[†]) in trifluoroacetic acid (3 mL) and dichloromethane (3 mL) was stirred for 4 h. The excess trifluoroacetic acid was neutralized by adding sodium hydroxide solution (2 mL, 2 M). The solvent was evaporated and the residue purified by flash chromatography eluting with 20% MeOH–EtOAc to afford **1** as a purple solid (60 mg, 66%). *R*_f 0.6 (EtOAc).

¹H NMR (500 MHz, DMSO-*d*₆) δ 8.03 (d, 1H, *J* = 10.0 Hz), 7.88 (s, 1H), 7.61 (d, 1H, *J* = 10.0 Hz), 7.27 (dd, 1H, *J* = 10.0, 5.0 Hz), 6.82 (dd, 1H, *J* = 10.0, 5.0 Hz), 6.73 (s, 1H), 6.19 (s, 1H), 4.88 (s, 2H), 4.08 (q, 4H, *J* = 5.0 Hz), 3.73 (t, 4H, *J* = 5.0 Hz), 2.63 (t, 4H, *J* = 5.0 Hz), 1.19 (t, 6H, *J* = 5.0 Hz).

¹³C NMR (125 MHz, DMSO-*d*₆) δ 182.2, 172.3, 170.6, 161.3, 152.4, 151.3, 146.9, 140.1, 134.1, 131.7, 128.1, 126.0, 124.9, 118.7, 111.1, 107.3, 105.1, 97.8, 65.6, 60.9, 47.1, 32.4, 14.9.

MS (ESI) *m/z* calculated for (M+H)⁺ 537.19 found 537.18 (M+H)⁺. *ν*_{cm⁻¹} (neat) 3389, 2954, 1721 cm⁻¹.

Synthesis of 2

The nitroso compound **B** (1.0 g, 3.1 mmol) and 1,7-dihydroxynaphthol (0.5 g, 3.1 mmol) were dissolved in dry DMF (20 mL) and heated to 140 °C for 4 h. The reaction mixture was cooled to 25 °C and DMF removed under reduced pressure. The residual material was purified by column chromatography eluting with 1/1 MeOH–EtOAc to afford **2** as a blue solid (0.94 g, 71%). *R*_f 0.3 (10% MeOH–EtOAc).

¹H NMR (500 MHz, CD₃OD) δ 8.55 (d, 1H, *J* = 8.8 Hz), 7.65 (d, 1H, *J* = 8.7 Hz), 7.56 (s, 1H), 7.23 (dd, 1H, *J* = 8.7, 2.7 Hz), 6.95 (dd, 1H, *J* = 8.7, 2.7 Hz), 6.74 (s, 1H), 6.35 (s, 1H), 3.66 (t, 2H, *J* = 8.3 Hz), 3.59 (q, 2H, *J* = 6.8 Hz), 2.92 (t, 2H, *J* = 8.3 Hz), 2.15 (br, 2H), 1.26 (t, 3H, *J* = 6.8 Hz).

¹³C NMR (125 MHz, CD₃OD) δ 184.3, 172.0, 159.9, 152.7, 151.3, 146.6, 138.9, 133.2, 130.7, 126.0, 125.9, 120.1, 110.9, 109.9, 104.3, 96.5, 49.4, 48.3, 45.2, 23.0, 11.5.

MS (ESI) m/z calculated for $(M-H)^-$ 427.10 found 427.02 $(M-H)^-$. $\nu_{cm^{-1}}$ (neat) 3397, 2931, 1689 cm^{-1} .

Synthesis of 3

2-Hydroxy diethyl Nile Red **D** (50.0 mg, 0.15 mmol) and K_2CO_3 (103.0 mg, 0.75 mmol) were dissolved in CH_3CN (5 mL). **13** (162.0 mg, 0.18 mmol) in CH_3CN (2 mL) was added dropwise in 5 min to the above solution at 25 °C. The reaction mixture was heated to 50 °C for 4 h. After completion of the reaction the solvent was evaporated and the residue was subjected to flash chromatography eluting with 30–40% acetone/EtOAc and then with 10% MeOH– CH_2Cl_2 to afford 150.0 mg of red colored material. Flash chromatography was performed to remove excess **13**.

The above red material (60.0 mg, 0.06 mmol) was dissolved in TFA/ CH_2Cl_2 (1/1, 3 mL) and stirred at 25 °C for 1 h. The solvent was removed under reduced pressure and the residual material was dissolved in 5 mL of water. This solution was filtered to remove solid impurities and the filtrate was purified by reverse phase medium pressure liquid chromatography (MPLC) eluting with 30% CH_3CN-H_2O to afford **3** as a dark red solid (35.0 mg, 58%).

¹H NMR (500 MHz, CD_3OD) δ 8.06 (d, 1H, $J = 8.9$ Hz), 8.01 (s, 1H), 7.97 (d, 1H, $J = 3.0$ Hz), 7.54 (d, 1H, $J = 8.9$ Hz), 7.17 (dd, 1H, $J = 9.2, 3.0$ Hz), 6.79 (dd, 1H, $J = 9.2, 3.0$ Hz), 6.54 (d, 1H, $J = 3.0$ Hz), 6.17 (s, 1H), 4.61 (s, 2H), 4.55 (t, 2H, $J = 5.0$ Hz), 4.32–4.29 (br, 2H), 3.93 (br, 2H), 3.89 (t, 2H, $J = 5.0$ Hz), 3.77–3.74 (br, 2H), 3.70–3.68 (br, 2H), 3.66–3.52 (m, 40H), 1.27 (t, 6H, $J = 7.0$ Hz).

¹³C NMR (75 MHz, CD_3OD) δ 183.7, 161.9, 152.8, 151.8, 147.1, 144.6, 143.8, 138.1, 134.3, 131.3, 127.3, 125.1, 124.7, 118.1, 110.7, 106.4, 103.7, 96.0, 72.5, 70.7, 70.5, 70.4, 70.3, 70.2, 70.1, 69.6, 69.2, 67.9, 63.8, 61.0, 50.2, 44.9, 11.8.

MS (ESI) m/z calculated for $(M+H)^+$ 944.49 found 944.49 $(M+H)^+$ **MS (ESI)** m/z calculated for $(M+Na)^+$ 966.47 found 966.45 $(M+Na)^+$; $\nu_{cm^{-1}}$ (neat) 3435, 2925, 1641 cm^{-1} .

Synthesis of 4

Compound **E** (25 mg, 0.06 mmol) and activating agent EDCI (28 mg, 0.24 mmol) were dissolved in pyridine (2 mL). Tris(hydroxymethyl)aminomethane (43 mg, 0.36 mmol) was added and the reaction was continued at 25 °C for 24 h. The solvent was evaporated and the residue purified by reverse phase medium pressure liquid chromatography (MPLC) eluting with 3/2 CH_3CN-H_2O to afford **4** as a dark red solid (22 mg, 59%).

¹H NMR (500 MHz, $DMSO-d_6$) δ 7.96 (d, 1H, $J = 10.0$ Hz), 7.87 (s, 1H), 7.61 (d, 1H, $J = 10.0$ Hz), 7.39 (s, 2H), 7.08 (dd, 1H, $J = 10.0, 5.0$ Hz), 6.84 (dd, 1H, $J = 10.0, 5.0$ Hz), 6.68 (s, 1H), 6.19 (s, 1H), 4.73 (br, 6H), 3.65 (br, 4H), 3.53 (s, 12H), 2.51 (br, 4H).

¹³C NMR (125 MHz, $DMSO-d_6$) δ 182.4, 172.1, 152.2, 152.2, 151.3, 146.9, 140.1, 134.4, 131.4, 128.2, 124.8, 119.2, 110.8, 110.2, 109.2, 105.2, 97.4, 63.2, 61.2, 48.1, 34.6.

MS (ESI) m/z calculated for $(M+H)^+$ 629.25 found 629.23 $(M+H)^+$. $\nu_{cm^{-1}}$ (neat) 3412, 3316, 1693 cm^{-1} .

Synthesis of 5

Compound **E** (60 mg, 0.14 mmol), N-hydroxysuccinimide (81 mg, 0.7 mmol) and N,N'-diisopropylcarbodiimide (88 mg, 0.7 mmol) were dissolved in dry DMF (2 mL) and stirred at 25 °C for 24 h. The solvent was evaporated under reduced pressure and the residue dissolved in EtOAc (5 mL) and washed with water (5 mL \times 3). The organic layer was dried over $MgSO_4$ and the solvent evaporated to obtain a red colored material.

The above red colored material was dissolved in DMF (2 mL) along with tricarboxylic acid **15** (479 mg, 1.4 mmol), DMAP (1 mg, 0.01 mmol) and triethylamine (0.2 mL, 1.4 mmol). The reaction mixture was stirred at 25 °C for 48 h. After removal of DMF under reduced pressure the residue was dissolved in water (2 mL) and washed with EtOAc (2 mL \times 3). The aqueous layer containing crude product was loaded on to a reverse phase MPLC column and purified using 3/2 CH_3CN-H_2O solvent mixture. The solvent was evaporated to afford **5** as a dark purple solid (43 mg, 28%).

¹H NMR (500 MHz, CD_3OD) δ 8.04 (br, 1H), 7.94 (br, 1H), 7.56 (br, 1H), 7.08 (br, 1H), 6.91 (s, 1H), 6.74 (s, 1H), 6.20 (s, 1H), 3.76 (br, 4H), 3.67 (br, 24H), 2.75 (br, 4H), 2.47 (s, 12H).

¹³C NMR (125 MHz, CD_3OD) δ 183.9, 175.6, 172.4, 161.0, 152.5, 151.3, 146.5, 139.1, 134.3, 130.8, 127.3, 125.1, 123.9, 118.0, 110.8, 108.4, 103.6, 96.9, 68.5 (2C), 67.2, 60.9, 35.4, 34.2.

MS (MALDI) m/z calculated for $(M+3H)^+$ 1063.39 found 1063.35 $(M+3H)^+$.

$\nu_{cm^{-1}}$ (neat) 2942, 1722, 1621 cm^{-1} .

Cell culture

Clone 9 cells (American Type Culture Collection) were cultured as subconfluent monolayers on 75 cm^2 culture flask with vent caps in Ham's medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5% CO_2 . Cells grown to subconfluence were enzymatically dissociated from the surface with trypsin and plated 2–3 days prior to the experiments in Lab-Tek two-well chambered coverglass slides (Nunc).

Fluorescence microscopy

Subcellular localization of Nile Red derivatives and BODIPY TR ceramide complexed to BSA was measured on living Clone 9 cells using a Stallion Dual Detector Imaging System consisting of an Axiovert 200M inverted fluorescence microscope, CoolSnap HQ digital cameras and Intelligent Imaging Innovations (3I) software. Digital images of Nile Red dyes, MITO tracker green labeled mitochondria, BODIPY TR ceramide complexed to BSA labeled Golgi, and LysoTracker Blue DND-22 were captured with a C-AP0 63X/1.2 W CORR $D = 0.28$ M27 objective with the following filter sets: Exciter BP560/40, Dichroic FT 585, Emission BP 630/75 for Nile Red derivatives and BODIPY TR ceramide complexed to BSA; Exciter BP470/20, Dichroic FT 493, Emission BP 505-530 for MITO tracker green; and Exciter G 365, Dichroic FT 395, Emission BP 445/50 for LysoTracker Blue DND-22.

Cellular uptake of Nile Red derivatives

Clone 9 cells were incubated for 30 min to one hour at 37 °C in ACAS with various concentration of probes (solution in PBS or DMSO). After the incubation period, the cells were washed

several times with phosphate-buffered saline (PBS, pH 7.4) before imaging (in ACAS). To identify the subcellular localization of the probe, the cells were costained with MITO tracker green (0.1 $\mu\text{L mL}^{-1}$), a mitochondria marker, or LYSO tracker blue (100 nM), a lysosome marker. A parallel experiment with BOD-IPY TR ceramide complexed to BSA was also carried out to confirm localization of Nile Red derivatives **4** and **D** in Golgi.

Measurement of the mitochondrial potential after treatment with **3**

Clone 9 cells were plated on a 96 well plated and allowed to grow for 2 days before treatment. Thereafter, the cells were incubated for 30 min with 1 μM of Nile red **3** at 37 °C, washed, then co-incubated with JC1 (5 $\mu\text{g mL}^{-1}$) for another 30 min.

As a control of fully depolarized mitochondria, cells were first treated with Nile Red **3**, washed, then co-incubated with JC1 (5 $\mu\text{g mL}^{-1}$) and 100 μM CCCP for an additional 30 min at 37 °C.

Mitochondrial membrane polarization was also measured on untreated cells. Thus, Clone 9 cells were treated with JC1 (5 $\mu\text{g mL}^{-1}$) for 30 min at 37 °C.

Depolarization of the mitochondrial membrane was also induced on a set of untreated cells. Thus, Clone 9 cells were treated with 100 μM CCCP for 30 min at 37 °C.

The cells were then analyzed on a BioTek Synergy 4 Microplate Reader using the Gen5 software. Fluorescence emission from JC-1 was measured looking at Exc. 485 nm, Emis. 530 nm; and Exc. 535 nm, Emis. 590 nm. The membrane potential was then obtained from the ratio red to green fluorescence from JC-1.

Intracellular calcium concentration

Clone 9 cells were incubated with 10 μM of **3** (stock soln in PBS) and 3 μM of Fluo4 in ACAS medium for 60 min at 37 °C. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.

In parallel, to estimate the intracellular concentration of calcium in Clone 9 cells (before treatment with **3**), Clone 9 cells were incubated with 3 μM of Fluo4 in ACAS medium for 60 min at 37 °C.

ROS concentration

Clone 9 cells were incubated with 10 μM of **3** (stock soln in PBS) and 2 $\mu\text{g mL}^{-1}$ of CM-H₂DCFDA in ACAS medium for 60 min at 37 °C. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.

In parallel, to estimate the intracellular concentration of cells were incubated with 2 $\mu\text{g mL}^{-1}$ of CM-H₂DCFDA in ACAS medium for 60 min at 37 °C.

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