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Synthesis of isomerically pure carboxylate- and sulfonate-substituted xanthene fluorophores

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Abstract—Xanthene-based fluorophores such as fluorescein and rhodamine are typically prepared by acid-catalyzed condensation of the appropriate resorcinol or 3-aminophenol with phthalic anhydride. Condensation of substituted phthalic anhydride species results in functionalized fluorophores that are formed as mixed isomers. Crystallization approaches to isomer separation have been reported elsewhere for symmetric fluorescein carboxylates. We describe crystallization-based separation of protected fluorescein sulfonates and coupling conditions to form sulfonamides, precursors for carboxylate-substituted rhodamines, and precursors for asymmetrically substituted fluoresceins and rhodafluors.

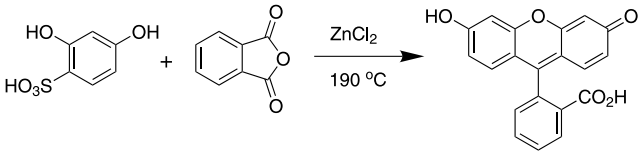
1. Introduction

Xanthene-based dyes such as rhodamines and fluoresceins are widely used in sensing applications owing to their brightness, high quantum yields, low-energy excitation and emission wavelengths, and biocompatibility. Functional handles are typically incorporated into the benzoic acid ring of these fluorophores. Bottom-ring-substituted rhodamines and fluoresceins form by acid-catalyzed condensation of a 3-aminophenol or resorcinol with a substituted phthalic acid species. This reaction affords the desired product as an equal mixture of two isomers, substituted at the 5- and 6-positions[1] of the benzoic acid ring. Crystallization-based separations of protected fluorescein carboxylate isomeric mixtures have been described,[1,2] and we now report similar methods in the separation of fluorescein 5(6) sulfonic acid isomers. However, there is no general method for separating rhodamine 5- and 6-carboxylate mixtures by crystallization. Chromatographic separation of isomers is particularly tedious with these polar, charged compounds.[3,4] Finally, asymmetrically substituted fluoresceins[5–8] and various hybrid rhodamine-fluorescein compounds,[9–13] termed rhodafluors or rhodols, have been prepared by our group and others. These compounds share a 2′,4′-dihydroxybenzophenone-2-carboxylate as a synthon, but the preparation of isomerically pure dicarboxy-substituted analogues of these benzophenones has not been reported. We now describe methodology for the synthesis of isomerically pure carboxylic or sulfonic acid-substituted analogues of these fluorophores.

2. Results and discussion

2.1. Fluorescein sulfonamides

In the course of our efforts to produce fluorescent sensors for Zn^{2+}, the synthesis of fluorescein sulfonamides was of interest owing to the many reported sulfonamide-based Zn^{2+} sensors. Sulfonation of the xanthene system was considered most likely to afford a derivative that would exhibit a metal-induced change in fluorescence. Direct sulfonation of unsubstituted fluorescein with fuming sulfuric acid provided only unreacted starting material, however. Reaction of sulfonated resorcinol with phthalic anhydride provided unsubstituted fluorescein as the major product (Scheme 1). Condensation of resorcinol with 4-sulfophthalic acid was explored next. As expected, this reaction produced a mixture of two isomers (**1a**, **b**, Scheme 2) in roughly equal amounts, a mixture that is available



Scheme 1.

Keywords: Fluorescein sulfonic acid; Rhodamine carboxylate; Isomer resolution; Fractional crystallization; Dibromofluoran; Asymmetric fluorescein carboxylate.
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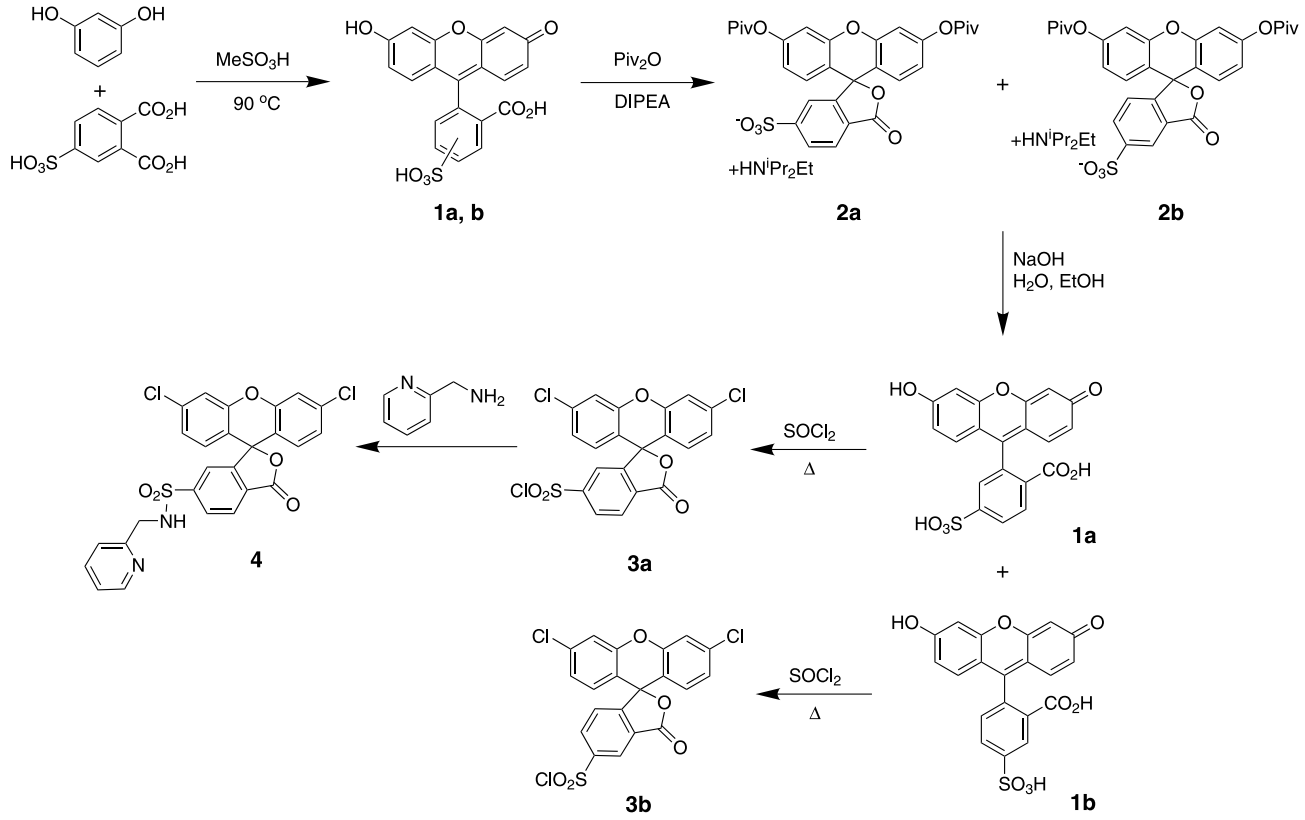


Scheme 2.

commercially.[14] The separation of fluorescein-5(6)-sulfonic acid isomers, reported previously, claimed isomeric purity based on infrared spectroscopy and paper chromatography.[15,16] The analytical sensitivity of these techniques is relatively low. We were able to separate the mixed isomers by protection as the dipivaloyl esters and subsequent crystallization of the 6-isomer **2a** followed by crystallization of the 5-isomer **2b**, each as its diisopropylethylammonium salt. Isomeric purity of **2a** and **2b** was greater than 95% based on NMR spectroscopy. Basic hydrolysis of the isomerically pure dipivaloates **2a** and **2b** yielded the deprotected fluorescein sulfonic acids **1a** and **1b**.

A crystal structure of fluorescein-6-sulfonic acid (**1a**) was obtained (Fig. 1). The crystal formed under strongly acidic conditions and, consequently, the fluorescein is in the

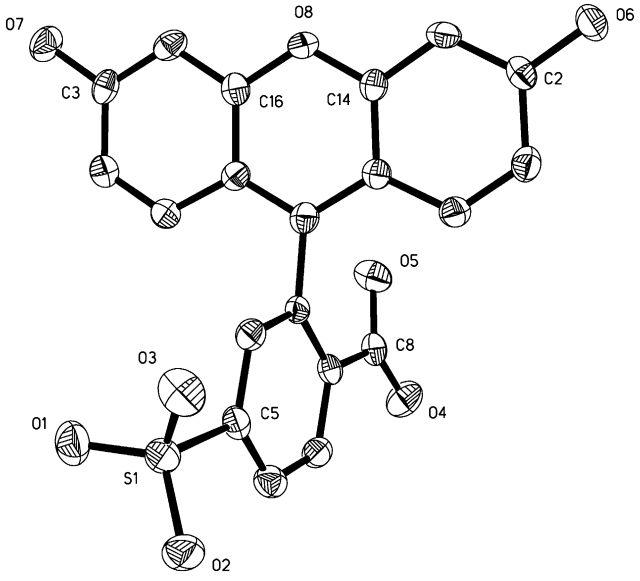


Figure 1. ORTEP diagram of fluorescein-6-sulfonic acid (**1a**) showing 50% probability thermal ellipsoids. The solvent molecules have been omitted for clarity.

Table 1. Crystallographic information

	1a·3H$_2$O	**4**
Empirical formula	$C_{20}H_{18}O_{11}S$	$C_{26}H_{16}N_2O_5SCl_2$
Molecular weight	466.40	539.37
Space group	$P2_1/c$	$P\bar{1}$
a (Å)	10.4324(12)	9.997(4)
b (Å)	16.854(2)	11.067(2)
c (Å)	11.9615(14)	11.401(2)
α, deg		95.65(3)
β, deg	109.516(2)	100.55(3)
γ, deg		106.81
V, Å^3	1982.3(4)	1171.6(4)
Z	4	2
ρ_{calc}, g/cm^3	1.563	1.529
T, °C	−85	−100
μ (Mo Kα), mm^{-1}	0.228	0.409
θ limits, deg	2.07–28.29	1.84–28.29
Total number of data	12393	10559
Number of unique data points	4568	5363
Number of parameters	316	352
R^a	0.0670	0.0433
wR^{2b}	0.1341	0.1070
max, min peaks, e/Å^3	0.464, −0.488	0.663, −0.199

[a] $R=\Sigma||F_o|-F_c||/\Sigma|F_o|$.
[b] $wR^2=\{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$.

lactone-opened form, presumably with a positive charge delocalized over the xanthene system. The charge is neutralized by the anionic sulfonate group. The C–O bonds of the phenolic hydroxyls are approximately the same length (1.34, 1.33 Å) and are each consistent with a single bond, rather than a ketone tautomer. Three water molecules were present in the asymmetric unit, with 12 in the unit cell. Crystallographic parameters are listed in Table 1 and selected bond lengths and angles for **1a**, in Table 2.

Table 2. Selected bond lengths and angles for **1a**

Bond lengths	(Å)	Bond angles	(Deg)
S(1)–O(2)	1.442(3)	O(2)–S(1)–O(1)	113.08(15)
S(1)–O(1)	1.451(3)	O(2)–S(1)–O(3)	112.75(17)
S(1)–O(3)	1.456(3)	O(1)–S(1)–O(3)	112.10(16)
S(1)–C(5)	1.780(3)	O(4)–C(8)–O(5)	124.7(3)
O(8)–C(14)	1.355(4)		
O(8)–C(16)	1.360(4)		
O(7)–C(3)	1.342(4)		
O(6)–C(2)	1.326(4)		
O(5)–C(8)	1.309(4)		
O(4)–C(8)	1.219(4)		

Coupling of the sulfonic acids with amines was first attempted following activation with thionyl chloride (Scheme 2). Reaction of both protected and unprotected fluoresceins under these conditions gave a non-fluorescent material **3a** or **3b**, however, which was determined to be the

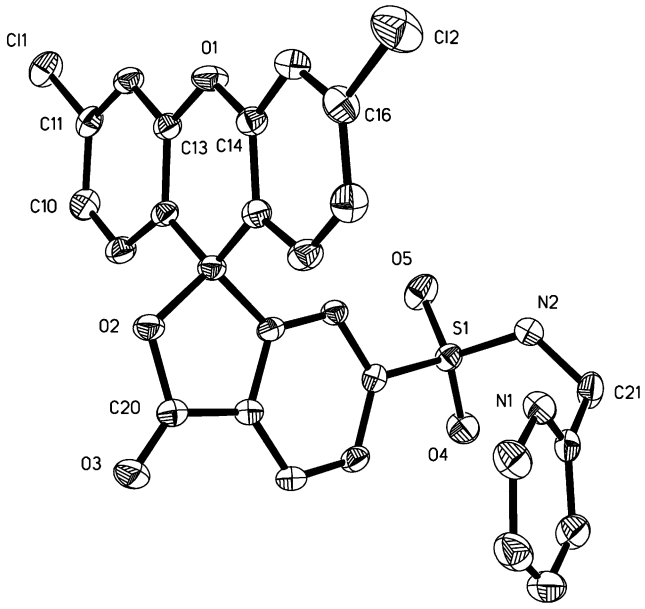


Figure 2. ORTEP diagram of fluorescein-6-sulfonamidopicoline (**4**) showing 50% probability thermal ellipsoids.

Table 3. Selected bond lengths and angles for **4**

Bond lengths	(Å)	Bond angles	(Deg)
Cl(1)–C(11)	1.7313(18)	O(5)–S(1)–O(4)	121.11(9)
Cl(2)–C(16)	1.7362(19)	O(5)–S(1)–N(2)	106.95(9)
S(1)–O(5)	1.4263(14)	O(4)–S(1)–N(2)	107.75(9)
S(1)–O(4)	1.4294(14)	C(21)–N(2)–S(1)	122.30(13)
S(1)–N(2)	1.5958(16)	O(3)–C(20)–O(2)	121.94(16)
O(1)–C(13)	1.371(2)	C(10)–C(11)–Cl(1)	119.33(14)
O(1)–C(14)	1.374(2)	C(13)–O(1)–C(14)	118.12(13)
N(2)–C(21)	1.456(2)		

3′, 6′-dichlorofluoran by X-ray crystallographic analysis of the 2-picolylamine adduct **4** (Fig. 2; selected bond lengths and angles listed in Table 3). The dihedral angle between the two extended aromatic ring systems is 86.6°, indicating a nearly perpendicular orientation. Use of the milder oxalyl chloride as an activating agent to generate sulfonyl chloride **5** and subsequent reaction with an amine affords the desired sulfonamide product as a mixture of unprotected (**6**) and protected (**7**) products (Scheme 3).

2.2. Rhodamines and rhodamine carboxylates

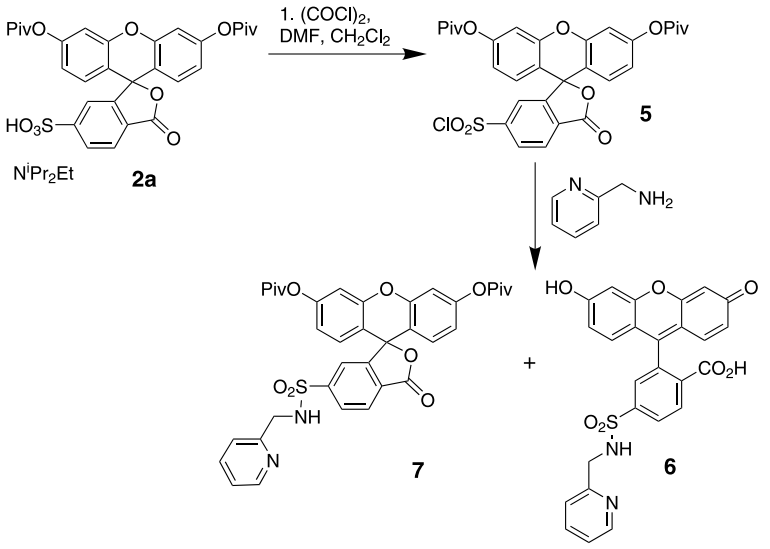
3′,6′-Dichlorofluoran can be converted into rhodamines by direct $ZnCl_2$-catalyzed condensation with excess amine.[17,18] This reaction suggested that, since 3′,6′-dihalofluorans share with 3′,6′-diacetyl- or -dipivaloyl-fluorescein the fluorescein motif that has been trapped in the lactone form, it might be possible to synthesize 3′,6′-dihalofluoran-5(6)-carboxylates by acid-catalyzed condensation and subsequently resolve the isomers by selective crystallization. Alternatively, reaction of previously resolved fluorescein carboxylates with thionyl chloride or thionyl bromide could provide the desired isomerically pure dihalofluoran carbonyl halide (vide supra). Subsequent condensation with amines under appropriate conditions might afford isomerically pure rhodamine carboxylates.[19]

We chose to work with the more reactive dibromofluoran species, rather than the reported dichlorofluran substrate. 3′,6′-Dibromofluoran **8** was synthesized by acid-catalyzed condensation of 3-bromophenol with phthalic anhydride and used as a model for the less readily available dibromofluoran carboxylates. 3′,6′-Dibromofluoran reacted smoothly with excess pyrrolidine under literature conditions to afford the desired rhodamine **9**. Palladium-catalyzed reductive coupling under reaction conditions described in the literature[20] also furnished **9**. This chemistry is summarized in Scheme 4.

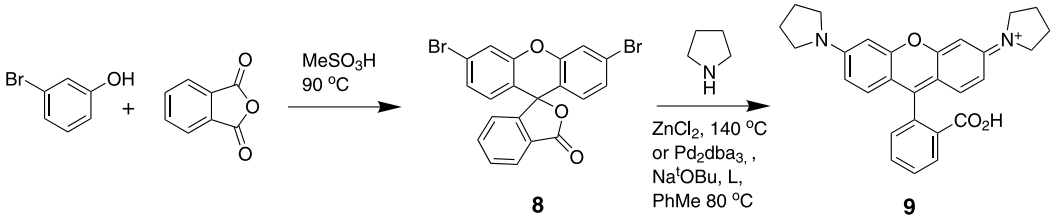
3′,6′-Dibromo-5(6)-carboxylate (**10a**, **b**) was synthesized similarly (Scheme 5). Selective crystallization from pyridine and acetic anhydride afforded the pure 6-isomer pyridinium salt **10c** in 15% yield, and further recrystallization of the mother liquor furnished the pure 5-isomer **10b** in 11% yield. Unsurprisingly, palladium-catalyzed coupling conditions were not compatible with a carboxylate-containing substrate. Nevertheless, heating **10c** at 140 °C with 5 equiv of $ZnCl_2$ and 10 equiv of pyrrolidine, followed by treatment with hydrochloric acid, afforded the desired rhodamine **11** in >94% yield with no apparent mixing of isomers. The strongly acidic workup is necessary in order to hydrolyze any amide byproduct that arises from carboxylate-amine condensation. The harsh conditions required for the reaction preclude the use of all but the most robust amines in the condensation.

2.3. Isomerically pure synthons for asymmetric carboxy-substituted xanthene dyes

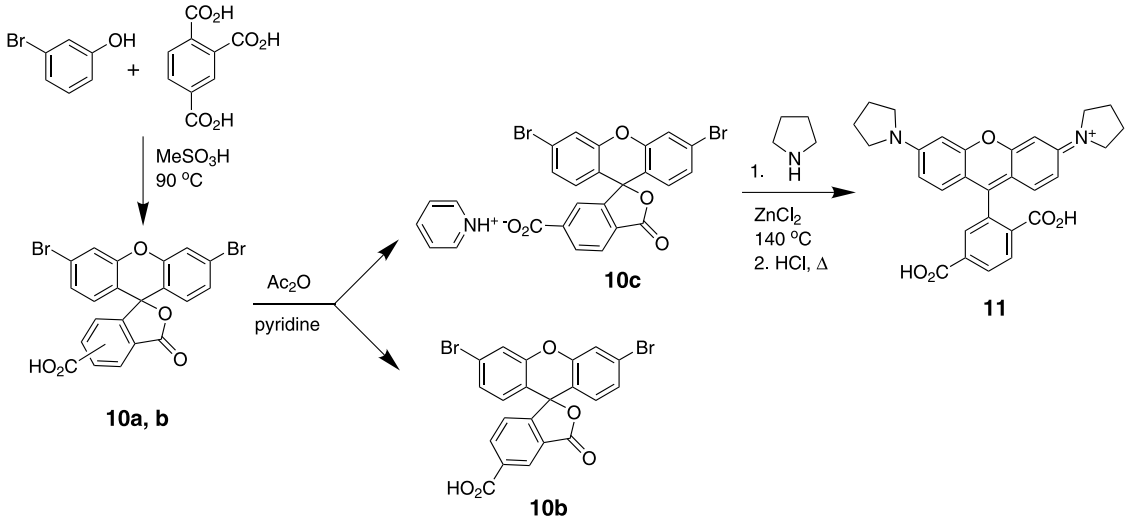
The syntheses of symmetrically substituted fluorescein-based zinc(II) biosensors containing a carboxylate or an amide functionality have been described previously.[21,22] To date, no such methodology exists for preparing similarly



Scheme 3.



Scheme 4.

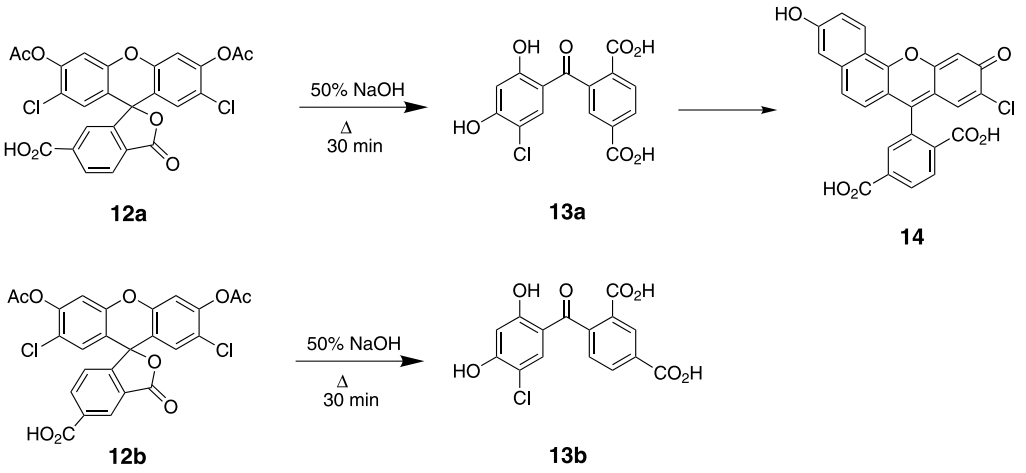


Scheme 5.

functionalized sensors based on asymmetric fluorescein scaffolds.[5,8,6] We, therefore, turned our attention to the synthesis of dihydroxydicarboxybenzophenones, which would be the logical starting material for the synthesis of asymmetric carboxylate-containing fluoresceins. Although subjecting 1,2,4-benzenetricarboxylic acid to aluminum chloride-catalyzed condensation with 4-chlororesorcinol[13] resulted in quantitative recovery of starting material, the hydrolysis of previously-resolved fluorescein carboxylates **12a** and **12b** under harshly basic conditions furnished the desired benzophenones **13a**, **13b** as isomerically pure compounds (Scheme 6). Results from condensation of **13a** with 1,6-dihydroxynaphthalene indicate that seminaphthofluorescein **14** can be obtained in excellent yield with no apparent scrambling of isomers.

3. Conclusions

We describe here the synthesis and separation of



Scheme 6.

fluorescein 5(6)-sulfonic acid. The structural assignment of fluorescein-6-sulfonic acid was confirmed in an X-ray crystallographic structure determination. Oxalyl chloride activation of the separated isomers affords the fluorescein sulfonyl chloride, whereas thionyl chloride converts the phenolic hydroxyls to chlorine atoms. Such dihalofluorans may also be synthesized by acid-catalyzed condensation of 3-halophenol with phthalic anhydride or analogues and can be converted to rhodamines via Lewis acid- or palladium-catalyzed reaction with simple amines. Carboxylate-substituted dihalofluorans are similar to diacetyl- or dipivaloyl-fluoresceins in that they are formed as a mixture of isomers, are trapped in lactone form, and may be separated by fractional crystallization. This methodology can be applied as a route to isomerically pure carboxylate-substituted rhodamines. Strongly basic hydrolysis of previously resolved fluorescein carboxylates affords the appropriate synthon for isomerically pure asymmetric fluorescein or rhodafluor carboxylates.

4. Experimental

4.1. Materials and methods

Reagents were obtained from Aldrich, except for 4-sulfophthalic acid, which was obtained from Lancaster, and palladium dibenzylideneacetone, sodium *tert*-butoxide, and 2′-dimethylamino-2-dicyclohexyl-phosphinobiphenyl, which were obtained from Strem. 4-Sulforesorcinol[23] and isomerically pure 3′,6-diacetyl-2′,7′-dichlorofluorescein-5(6)-carboxylates[22] were prepared as previously described. The purity of all compounds reported here was judged to be >95% by NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a 300 or 500 MHz Varian or 400 MHz Bruker instrument. HRMS data were acquired by personnel at the MIT DCIF. Low-resolution electrospray mass spectra were obtained on an Agilent Technologies 1100 Series LCMS. Single crystals suitable for X-ray crystallography were covered with Infineum V8512 (formerly called Paratone N oil) and mounted on a quartz fiber. Data were collected by using a Bruker CCD X-ray diffractometer with Mo Kα radiation (λ=0.71073 Å) using the SMART software package[24] and corrected for absorption using SADABS v 6.2.[25] Data were integrated using the SAINT-PLUS software package,[26] and structures were solved and refined using SHELXTL.[27] Procedures for data collection and structural work have been reported in detail elsewhere.[28] All non-hydrogen atoms were refined anisotropically using least-squares methods and Fourier syntheses. Hydrogen atoms were assigned idealized positions and given thermal parameters of 1.2 times the thermal parameter of the carbon or nitrogen atom to which they were attached. All structure solutions were checked for higher symmetry with the PLATON program.[29]

4.2. Synthetic procedures

4.2.1. Condensation of 4-sulforesorcinol with phthalic anhydride. 4-Sulforesorcinol (424 mg, 2 mmol) was ground together with phthalic anhydride (148 mg, 1 mmol) and $ZnCl_2$ (41 mg, 0.3 mmol) with a mortar and pestle, and the resulting mixture was heated in a 190 °C oil bath for 30 min. Water and MeOH were added and the resulting solution was extracted with 3×20 mL portions of CH_2Cl_2. The combined organic layers were washed with 1×20 mL of brine, dried over $MgSO_4$, and evaporated to afford a dark orange-brown solid residue. ^{1}H NMR analysis indicated unsubstituted fluorescein as the major product. ^{1}H NMR (MeOH-d_4): δ 8.05 (d, 1H, J=7.5 Hz); 7.77 (td, 1H, J=7.5, 2.1 Hz); 7.70 (td, 1H, J=7.5, 2.1 Hz); 7.21 (d, 1H, J=7.5 Hz); 6.70 (d, 2H); 6.60 (d, 2H, J=3.2 Hz); 6.61–6.52 (m, 4H).

4.2.2. Fluorescein-5(6)-sulfonic acid (1). A 1.14 mL portion of a 50% aqueous solution of 4-sulfophthalic acid (0.736 g, 3 mmol, 80% pure) was neutralized with potassium hydroxide (45% w/v in water) and the solvent was evaporated to give a purple semi-solid residue, which was combined with resorcinol (0.661 g, 6 mmol) in 6 mL of methanesulfonic acid. The reaction mixture was stirred in an 85 °C oil bath for 18 h, then poured into 40 mL of H_2O. A reddish-brown precipitate was filtered and dried under vacuum to afford 696 mg of a brown powder (71% yield), which was carried on without further purification. ^{1}H NMR (MeOH-d_4): δ 8.81 (s, 1H, J=1.8 Hz); 8.46 (d, 1H, J=8.1 Hz); 8.23–8.30 (m, 2H); 7.84 (d, 1H, J=1.8 Hz); 7.5 (d,

1H, $J=8.1$ Hz); 7.5 (s, 2H); 7.46 (s, 2H); 7.36 (t, 4H, $J=3.0$ Hz); 7.2 (m, 4H). MS(M-H): calcd 411.0; Found 411.4.

4.2.3. Diisopropylethylammonium salt of 3′,6′-dipivaloylfluorescein-6-sulfonate (2a). Fluorescein-5(6)-sulfonic acid (9.08 g, 22 mmol) was dissolved in 35 mL of trimethylacetic anhydride, 17.5 mL of diisopropylethylamine, and 30 mL of DMF. The solution was heated to reflux for 4 h and then quenched by addition of ethanol. The solvents were removed on the rotary evaporator and the resulting light brown viscous oil was taken up in 200 mL of CH_2Cl_2 and 400 mL of ethyl acetate, and washed with 3× 300 mL of 1 M phosphate buffer (pH 7.0). The organic phase was dried over $MgSO_4$ and evaporated. Diethyl ether was added, and a heavy fine precipitate formed. The filtered solid was recrystallized from dichloromethane and diethyl ether to give 2.10 g (13.5%) of fluorescein 6-sulfonic acid dipivaloate diisopropylethylammonium salt; mp 227–229 °C. ^{1}H NMR ($CDCl_3$): δ 9.00 (br s, 1H); 8.17 (d, 1H, $J=8.1$ Hz); 8.04 (d, 1H, $J=8.1$ Hz); 7.64 (s, 1H); 7.04 (d, 2H, $J=2.1$ Hz); 6.84 (d, 2H, $J=8.7$ Hz); 6.76 (dd, 2H, $J=8.7$, 2.1 Hz); 3.60 (m, 2H); δ 3.03 (m, 2H); 1.36 (s, 18H); 1.30–1.34 (m, 15H). ^{13}C NMR ($CDCl_3$): δ 176.8, 169.6, 154.1, 152.6, 151.6, 147.0, 131.4, 129.1, 125.1, 124.4, 123.4, 117.8, 116.6, 110.4, 81.4, 39.4, 27.3, 22.3. HRMS(M-HN^{i}Pr$_2$Et): calcd 579.1325; Found 579.1330.

4.2.4. Diisopropylethylammonium salt of 3′,6′-dipivaloylfluorescein-5-sulfonate (2b). The filtrate from **2a** was allowed to stand at room temperature overnight, and the resulting light yellow crystals were filtered to give 0.91 g (5.8% overall) of the diisopropylethylammonium salt of fluorescein 5-sulfonic acid dipivaloate; mp 154–156 °C. ^{1}H NMR ($CDCl_3$): δ 9.25 (br s, 1H); 8.33 (s, 1H); 8.06 (d, 1H, $J=6.6$ Hz); 6.97 (d, 1H, $J=8.1$ Hz); 6.86 (d, 2H, $J=1.8$ Hz); 6.61–6.56 (m, 4H); 3.56 (m, 2H); 2.98 (m, 2H); 1.35 (m, 9H); 1.26 (d, 6H, $J=6.6$ Hz); 1.17 (s, 18H). ^{13}C NMR (DMSO-d_6): δ 176.0, 168.1, 152.4, 152.2, 150.8, 150.8, 133.4, 129.3, 125.2, 123.9, 121.5, 118.5, 115.9, 110.4, 81.1, 53.6 41.9, 38.7, 27.0, 26.7, 18.1, 16.7, 12.5. MS(M-HN^{i}Pr$_2$Et): Calcd 579.1; Found 579.2. Continued fractional crystallization brought the final yields to 3.31 g, 21% for the 6-isomer; and 1.59 g, 10.1% for the 5-isomer.

4.2.5. Optimized purification of diisopropylethylammonium salt of 3′,6′-dipivaloyl-fluorescein-6-sulfonate (2a). Fluorescein 5(6)-sulfonic acid (6.20 g, 15 mmol) was combined with trimethylacetic anhydride (35 mL) and diisopropylethylamine (18 mL) in DMF (20 mL) and heated to reflux 4 h. The reaction was allowed to cool, then taken up in 200 mL of CH_2Cl_2 and 400 mL of ethyl acetate. The organic solution was washed with 3×300 mL of phosphate buffer (1 M, pH 7.0), dried over $MgSO_4$, evaporated, and the resulting residue was crystallized from diethyl ether and CH_2Cl_2 at −25 °C overnight. Filtration gave 2.49 g (23.4%) of the desired product. The mother liquor was placed in the freezer and a second crop of crystals was obtained (800 mg) for a total yield of 3.29 g (31% overall).

4.2.6. Fluorescein 6-sulfonic acid (1a). The diisopropylethylammonium salt of 3′,6′-dipivaloylfluorescein 6-sulfonic acid (177 mg, 0.25 mmol) was dissolved in 10 mL of 50:50 v/v ethanol/H_2O. Potassium hydroxide was added (0.40 g) and the reaction was heated to reflux overnight. The ethanol was then removed under reduced pressure, the dark red solution was acidified, and the resulting orange precipitate (71 mg, 69%) was collected by filtration and dried overnight; mp >338 °C (decomp.). ^{1}H NMR (DMSO-d_6): δ 7.95 (d, 1H, $J=7.8$ Hz); 7.87 (d, 1H, $J=9.3$ Hz); 7.25 (s, 1H); 6.70 (s, 2H); 6.55–6.62 (m, 4H). IR: 3400–2500 cm^{-1} (br, s), 1707 cm^{-1} (m), 1638 cm^{-1} (m), 1603 cm^{-1} (s), 1457 cm^{-1} (s), 1314–1122 cm^{-1} (br, s), 1037 cm^{-1} (s). ^{13}C NMR (DMSO-d_6): δ 168.4, 160.0, 154.9, 152.0, 129.3, 127.7, 125.9, 125.0, 120.6, 113.1, 109.5, 102.3. HRMS(M+H): Calcd 413.0331; Found 413.0320. The acidic filtrate obtained was allowed to stand at RT for 2 weeks, at the end of which time small bright-orange X-ray quality crystals of **1a** had formed.

4.2.7. 3′,6′-Dichlorofluoran-6-sulfonyl chloride (3a). Fluorescein 6-sulfonic acid (412 mg, 1 mmol) was added to thionyl chloride (2.6 mL, 6 g, 5 mmol) and dimethylformamide (6 mg, 6 μL, 82 μmol) and the reaction was heated to reflux under Ar for 4 h. The resulting solution was poured into 150 mL of stirred ice water and stirred for an additional 10 min. The yellow grainy solid obtained was lyophilized to give a final mass of 360 mg (87% yield); mp >210 °C (decomp.). ^{1}H NMR (DMSO-d_6): δ 8.02 (d, 1H, $J=8.1$ Hz); 7.94 (d, 2H, $J=1.2$ Hz); 7.92 (d, 1H, $J=2.4$ Hz); 7.58 (s, 2H); 7.39 (s, 1H); 7.22 (dd, 2H, $J=8.6$, 1.8 Hz), 6.94 (d, 2H, $J=8.4$ Hz). ^{13}C NMR (DMSO-d_6): δ 168.1, 155.6, 152.6, 150.6, 135.4, 129.9, 128.3, 125.3, 125.0, 124.9, 120.5, 117.4, 117.1, 80.3. FTIR (KBr, cm^{-1}): 3422 (br, m), 1777 (s), 1599 (m), 1566 (w), 1482 (m), 1411 (s), 1266–1060 (br, s), 955 (m). MS(M–Cl+OH): Calcd 446.9; Found 447.0.

4.2.8. Fluorescein 5-sulfonic acid (1b). 3′,6′-Dipivaloylfluorescein 5-sulfonic acid diisopropylethyl ammonium salt (1.42 g, 2 mmol) and potassium hydroxide (3.2 g) were dissolved in 30 mL of 50:50 v/v ethanol/H_2O and heated to reflux overnight. The ethanol was removed under reduced pressure, and the aqueous solution was acidified with concentrated HCl, causing the product to precipitate. Filtration and drying overnight afforded the desired product as 696 mg (84%) of a yellow solid; mp >330 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ 8.06 (s, 1H); 7.98 (d, 1H, $J=9.3$ Hz); 7.22 (d, 1H, $J=8.1$ Hz); 6.67 (d, 2H, $J=2.1$ Hz); 6.61–6.55 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 168.3, 160.3, 152.3, 150.2, 133.0, 129.5, 126.2, 124.3, 121.6, 113.2, 109.8, 102.3. FTIR (KBr, cm^{-1}): 3500–2500 (br, s), 1714 (s), 1639–1538 (br, s) 1463 (s), 1383 (m), 1326 (s), 1220–1126 (s), 1039 (m). HRMS(M-H): Calcd 411.0175; Found 411.0155.

4.2.9. 3′,6′-Dichlorofluoran-5-sulfonyl chloride (3b). Fluorescein 5-sulfonic acid (618 mg, 1.5 mmol) was combined with thionyl chloride (4 mL) and dimethylformamide (10 μL) and heated to reflux for 4 h. The reaction was poured into 150 mL of stirred ice water, affording a dark yellow solid that was filtered and lyophilized to give a final mass of 549 mg (82% yield); mp >232 °C (decomp.). ^{1}H NMR (DMSO-d_6): δ 8.10 (s, 1H); 8.00 (d, 1H, $J=9.6$ Hz); 7.58 (d, 2H, $J=2.1$ Hz); 7.33 (d, 1H, $J=8.7$ Hz); 7.20 (dd, 2H, $J=8.6$, 2.4 Hz); 6.95 (d, 2H, $J=8.4$ Hz). ^{13}C NMR (DMSO-d$_6$): δ 168.1, 152.3, 150.9, 150.7, 135.4, 133.6,

130.1, 125.0, 124.0, 121.7, 117.5, 117.1, 80.4. IR: 3091 cm^{-1} (br, w), 1779 cm^{-1} (s), 1599 cm^{-1} (s), 1564 cm^{-1} (s), 1481 cm^{-1} (s), 1425–1384 cm^{-1} (br, s), 1318 cm^{-1} (m), 1251–1083 cm^{-1} (br, m), 954 cm^{-1} (s). HRMS(M–Cl+OH): Calcd 446.9497; Found 446.9503.

4.2.10. 3′, 6′-Dichlorofluoran-6-sulfonamido-2-methylpyridine (4). 3′,6′-Dichlorofluoran-6-sulfonyl chloride (**4**, 86 mg, 0.2 mmol) was dissolved in 15 mL of CH_2Cl_2 and added dropwise to a stirred suspension of 2-aminomethylpyridine (43 mg, 0.4 mmol) and $NaHCO_3$ (84 mg, 1 mmol) in CH_2Cl_2. After stirring overnight, the reaction suspension was filtered and evaporated; the product was purified by flash chromatography on silica gel (10 mm×17 cm) eluting with 9:1 $CHCl_3$:MeOH, and then recrystallized from methanol to give 30 mg (30%) of an off-white powder; mp 236–238 °C. ^{1}H NMR (DMSO-d_6): δ 8.59 (t, 1H, J=5.7 Hz), 8.31 (d, 1H,, J=4.8 Hz), 8.20 (d, 1H, J=8.4 Hz), 8.08 (dd, 1H, J=8.7, 0.6 Hz), 7.76 (s, 1H), 7.66 (td, 1H, J=7.8, 2.1 Hz), 7.61 (d, 2H, J=1.8 Hz), 7.15–7.26 (m, 3H), 6.91 (d, 2H, J=8.4 Hz), 4.12 (d, 2H, J=6.0 Hz). ^{13}C NMR (DMSO-d_6): δ 168.2, 157.5, 153.6, 151.8, 149.8, 149.3, 137.9, 136.7, 131.1, 130.0, 129.3, 127.6, 126.1, 123.7, 123.2, 122.8, 118.2, 118.0, 81.9, 48.9. MS(M-H): Calcd 537.0; Found 537.1. X-ray quality crystals were obtained by slow evaporation of a saturated acetonitrile solution of **4** at RT over 2 days.

4.2.11. 3′,6′-Dipivaloylfluorescein-6-sulfonyl chloride (5). 3′,6′-Dipivaloylfluorescein-6-sulfonate diisopropylethylammonium salt (708 mg, 1 mmol) was stirred in 10 mL of ethyl acetate (dried over $MgSO_4$) in an ice bath. Oxalyl chloride (1 mL of 2 M solution in CH_2Cl_2) was added, followed by 200 μL of DMF. The ice bath was removed, and the reaction was stirred for 16 h. The reaction was then placed on ice and quenched with 10 mL of H_2O. The layers were separated, and the organic layer was washed with 1×10 mL H_2O and 1×10 mL brine, dried, and evaporated to give a yellow solid residue; mp >160 °C (decomp.). ^{1}H NMR ($CDCl_3$): δ 8.68 (s, 1H); 8.31 (d, 1H, J=6.8 Hz); 7.45 (d, 1H, J=6.5 Hz); 7.18 (s, 2H); 6.84 (s, 2H); 1.37 (s, 18H). HRMS(M+H): Calcd 599.1143; Found 599.1114.

4.2.12. 6-Fluoresceinsulfonamido-2-methylpyridine (6). The product from **5** was dissolved in 20 mL of $CHCl_3$ and stirred in an ice bath. 2-Aminomethylpyridine (300 μL) was added and the reaction was stirred overnight. The reaction was then extracted with 2×20 mL of H_2O, the combined aqueous layers were washed with 1×20 mL $CHCl_3$, concentrated on the rotary evaporator to 5 mL, and the bright red viscous solution was acidified with 2 mL of 1 N HCl. The resulting yellow precipitate was filtered and the resulting solid (485 mg) was chromatographed on silica (20 mm×16 cm), eluting with 89:10:1 $CHCl_3$:MeOH:AcOH. The desired product (43 mg, 8.5%) was isolated as a bright yellow–orange solid; mp 78–80 °C. ^{1}H NMR ($CDCl_3$): δ 8.40–8.36 (m, 2H); 8.10 (dd, 1H, J=8.1, 1.5 Hz); 7.74 (td, 1H, J=7.8, 1.5 Hz); 7.43 (d, 1H, J=7.8 Hz); 7.31–7.23 (m, 2H); 6.69 (t, 2H, J=0.9 Hz); 6.59 (s, 4H); 4.34 (s, 2H). ^{13}C NMR (DMSO-d_6): δ 167.4, 156.7, 152.4, 148.8, 142.8, 136.8, 136.5, 129.5, 129.3, 122.5, 122.4, 122.0, 109.1, 102.5, 102.3, 48.0. HRMS(M+H): Calcd 503.0913; Found 503.0912. The dipivaloyl-protected sulfonamide product **7** was also isolated from the organic reaction extract by flash chromatography on silica eluted with 94:6 $CHCl_3$:MeOH; mp 184–186 °C. ^{1}H NMR (MeOH-d_4): δ 8.38 (m, 2H); 8.09 (dd, 1H, J=8.1, 1.8 Hz); 7.61 (td, 1H, J=7.8, 2.1 Hz); 7.25 (s, 1H); 7.21–7.12 (m, 3H), 7.05 (d, 1H, J=2.1 Hz); 6.84–6.77 (m, 3H); 6.70 (d, 2H, J=8.4 Hz); 4.40 (s, 2H); 1.36 (s, 18H). ^{13}C NMR (DMSO-d_6): δ 176.1, 167.1, 156.5, 155.0, 152.7, 150.9, 148.8, 143.6, 136.8, 133.7, 129.4, 126.1, 125.2, 123.5, 122.6, 122.2, 118.7, 115.3, 110.5, 81.4, 48.0, 38.8, 26.7. HRMS (M+H): Calcd 671.2063; Found 671.2071.

4.2.13. 3′,6′-Dibromofluoran (8). 3-Bromophenol (1.73 g, 10 mmol) and phthalic anhydride (740 mg, 5 mmol) were combined in 5 mL of methanesulfonic acid and heated in a 140 °C oil bath for 16 h. The reaction was poured into 120 mL of stirred ice water, stirred for 20 min, and then filtered. The resulting damp gray solid was taken up in $CHCl_3$ and filtered through a short plug of silica gel, evaporated, and recrystallized from CH_2Cl_2 and MeOH to afford the desired product as 990 mg of off-white crystals (43% yield); mp 277–280 °C. ^{1}H NMR ($CDCl_3$): δ 8.05 (dd, 1H, J=7.8, 1.5 Hz); 7.67 (p, 2H, J=1.2 Hz); 7.50 (d, 2H, J=1.8 Hz); 7.20 (dd, 2H, J=10.5, 2.1 Hz); 7.14 (d, 1H, J=7.8 Hz); 6.71 (d, 2H, J=8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 169.4, 153.2, 151.5, 135.9, 130.7, 129.6, 127.9, 126.2, 125.9, 124.6, 124.1, 120.8, 118.3, 81.5. HRMS(M+H): Calcd 456.9075; Found 456.9084.

4.2.14. 3′,6′-Pyrrolidinorhodamine (9)—method A. 3′,6′-Dibromofluoran (46 mg, 0.1 mmol) was combined with $ZnCl_2$ (68 mg, 0.5 mmol) and pyrrolidine (83 μL, 71 mg, 1 mmol) and heated in a 170 °C oil bath for 4 h. The reaction was removed from heat, and allowed to cool. Water and concentrated HCl were added; the suspension was stirred, filtered, and the solid was washed twice with dilute HCl to afford a purple solid (42 mg, 95% yield); mp >220 °C (decomp.). ^{1}H NMR (MeOH-d_4): δ 8.34 (d, 1H, J=7.5 Hz); 7.81 (m, 2H); 7.41 (d, 1H, J=8.7 Hz); 7.12 (d, 2H, J=9.3 Hz); 6.90 (dd, 2H, J=6.9, 1.8 Hz); 6.82 (d, 2H, J=2.1 Hz); 3.61 (m, 8H); 2.14 (s, 8H). ^{13}C NMR (DMSO-d_6): δ 169.0, 152.7, 152.3, 152.3, 149.2, 135.3, 129.8, 128.9, 128.5, 128.3, 126.7, 125.4, 124.5, 124.0, 108.8, 105.4, 97.4, 85.4, 47.4, 25.0. MS(M+H): Calcd 439.2; Found 439.4.

4.2.15. 3′,6′-Pyrrolidinofluoran (9)—method B. 3′,6′-Dibromofluoran (229 mg, 0.5 mmol) was combined with palladium dibenzylideneacetone (11.5 mg, 0.0125 mmol; 0.025 mmol Pd), sodium *tert*-butoxide (101 mg, 1.05 mmol), and 2′-dimethylamino-2-dicyclohexyl-phosphinobiphenyl (10.3 mg, 0.025 mmol) in a thick-walled tube fitted with a rubber septum. The tube was thrice evacuated and back-filled with N_2, and 1.5 mL of dry toluene was added, followed by 90 μL (77 mg, 1.08 mmol) of pyrrolidine. The septum was replaced with a Teflon screw cap and the reaction was stirred in an 80 °C oil bath for 15 h, then removed from heat and allowed to cool. Hexanes were added to the purple slurry, and a purple solid was isolated by filtration (350 mg, wet) LCMS and ^{1}H NMR analysis of the solid supported a single product identical to those produced by method A.

4.2.16. Pyridinium salt of 3′,6′-dibromo-6-carboxyfluoran (10c). 3-Bromophenol (3.46 g, 20 mmol) and 1, 2, 4-benzenetricarboxylic acid (2.10 g, 10 mmol) were combined in 10 mL of methanesulfonic acid and heated in a 140 °C oil bath for 3 days. The reaction was poured into 200 mL of stirred ice water, stirred vigorously with warming for 30 min, and then filtered to yield a greenish solid which was dried in air to give 3.83 g of **10a** and **10b** as a mixture of isomers. Crystallization from 30 mL of acetic anhydride and 10 mL of pyridine afforded 1.35 g of white solid, which was recrystallized from 2:1 Ac_2O:pyridine to furnish the desired compound **10c** as 1.07 g (18%) of fine white crystals; mp >327 °C (decomp.). ^{1}H NMR (DMSO-d_6): δ 8.58 (m, 2H); 8.25 (d, 1H, J=6.5 Hz); 8.16 (d, 1H, J=6.4 Hz); 7.86 (s, 1H); 7.76 (m, 1H); 7.43 (m, 2H); 6.87 (d, 2H, J=6.4 Hz); 6.67 (d, 2H, J=6.3 Hz). ^{13}C NMR (DMSO-d_6): δ 172.1, 167.6, 166.0, 152.2, 150.8, 149.6, 137.9, 136.2, 131.5, 130.1, 128.6, 127.7, 125.7, 124.8, 124.0, 123.8, 119.9, 117.5, 80.8. HRMS(M-pyH): Calcd 498.8817; Found 498.8804.

4.2.17. 3′,6′-Dibromo-5-carboxyfluoran (10b). The filtrate from the initial crystallization of **10c** was concentrated and recrystallized from pyridine to afford 906 mg of an off-white solid. Further recrystallization from $CHCl_3$:MeOH afforded the desired product as 571 mg of white crystals (11.4% yield); mp >324 °C (decomp.). ^{1}H NMR (DMSO-d_6): δ 8.44 (s, 1H); 8.31 (d, 1H, J=8.0, 1.2 Hz); 7.72 (d, 2H, J=2.0 Hz); 7.51 (d, 1H, J=8.0 Hz); 7.34 (dd, 2H, J=8.6, 2.0 Hz); 6.90 (d, 2H, J=8.4 Hz). ^{13}C NMR (DMSO-d_6): δ 167.5, 165.9, 155.5, 150.6, 136.5, 133.4, 130.8, 127.8, 127.7, 126.0, 125.9, 124.6, 123.9, 119.9, 117.4, 80.6. MS(M+H): Calcd 500.9; Found 501.0.

4.2.18. 3′,6′-Dipyrrolidino-6-carboxyrhodamine (11). 3′,6′-Dibromo-6-carboxyfluoran (116 mg, 0.2 mmol) was combined with $ZnCl_2$ (136 mg, 1 mmol) and pyrrolidine (332 μL, 5 mmol) and heated in a 140 °C oil bath for 4 h. The dark purple residue was dissolved in 15 mL of concentrated HCl, and the resulting dark red solution was filtered and then diluted with 30 mL of H_2O, allowed to stand at rt for 2 h, and filtered to yield 98 mg (94%) of the desired product HCl salt; mp >310 °C (decomp.). ^{1}H NMR (MeOH-d_4): δ 8.42 (s, 1H); 8.39 (d, 1H, J=5.8 Hz); 7.98 (s, 1H); 7.11 (d, 2H, J=6.4 Hz); 6.94–6.84 (m, 4H); 3.62 (br s, 8H); 2.14 (s, 8H). HRMS(M-H): Calcd 481.1763; Found 481.1744.

4.2.19. 2,5-Dicarboxy-5′-chloro-2′,4′-dihydroxybenzophenone (13a). 3′,6′-Diacetyl-2′,7′-dichlorofluorescein-6-carboxylic acid pyridinium salt (2.44 g, 4 mmol) was suspended in 60 mL of 50% aqueous NaOH (w/v) and heated at 165 °C for 60 min. The reaction was removed from the heating bath, poured into 400 mL of cold H_2O, acidified with conc HCl, and allowed to stand at rt for 2 h. The suspension was filtered, and the pale yellow solid was taken up in MeOH, filtered to remove residual NaCl, and evaporated to afford 1.19 g of the desired product (89% yield); mp >250 °C (decomp.). ^{1}H NMR (MeOH-d_4): δ 8.20 (m, 2H); 7.98 (s, 1H); 6.97 (s, 1H); 6.49 (s, 1H). ^{13}C NMR (MeOH-d_4): δ 201.2, 168.0, 168.0, 164.8, 162.0, 141.6, 135.7, 134.6, 134.5, 132.1, 132.0, 129.6, 115.4, 113.5, 105.0. MS(M-H): Calcd 335.0; Found 335.0.

4.2.20. 2,4-Dicarboxy-5′-chloro-2′,4′-dihydroxybenzophenone (13b). 3′,6′-Diacetyl-2′,7′-dichlorofluorescein-5-carboxylic acid (2.12 g, 4 mmol) was suspended in 60 mL of 50% aqueous NaOH (w/v) and heated at 165 °C for 60 min. The reaction was removed from heat, poured into 400 mL of cold H_2O, acidified with conc. HCl, and allowed to stand at rt overnight. The suspension was filtered, and the dirty-brown solid was resuspended in 50 mL of H_2O, stirred, and filtered again. The resulting solid was then taken up in MeOH, filtered to remove residual NaCl, and evaporated to afford 1.14 g of the desired product (85% yield); mp >265 °C (decomp.). ^{1}H NMR (MeOH-d_4): δ 8.72 (s, 1H); 8.34 (d, 1H, J=5.7 Hz); 7.51 (d, 1H, J=4.8 Hz); 6.95 (s, 1H); 6.49 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 201.4, 168.1, 167.9, 164.8, 162.0, 145.2, 134.7, 134.6, 133.8, 132.9, 131.2, 129.1, 115.4, 113.6, 105.0. HRMS(M-H): Calcd 334.9959; Found 334.9944.

4.2.21. 6-Carboxy-2′-chloroseminaphthofluorescein (14). 1,6-Dihydroxynaphthofluorescein (24 mg, 0.15 mmol) was combined with benzophenone **13a** (34 mg, 0.1 mmol) in 200 μL of methanesulfonic acid in a 180 °C oil bath. After 14 h, 5 mL of H_2O was added and the resulting suspension was filtered and washed twice with H_2O. The resulting purple solid was dissolved in MeOH, the solution was filtered, and H_2O was added to the filtrate to induce precipitation. The product was collected by filtration and dried in air to give 42 mg (91%) of the desired compound; mp >256 °C (sublimed). ^{1}H NMR (DMSO-d_6): δ 11.2 (br s, 1H); 10.2 (br s, 1H); 8.40 (d, 1H, J=8.8 Hz); 8.24 (d, 1H, J=1.2 Hz); 8.16 (d, 1H, J=8.0 Hz); 7.72 (s, 1H); 7.38 (d, 1H, J=8.8 Hz); 7.27 (d, 1H, J=9.0 Hz); 7.15 (s, 1H); 7.14 (s, 1H); 6.91 (s, 1H); 6.62 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 167.9, 166.1, 157.5, 155.2, 152.7, 145.0, 146.4, 137.5, 136.0, 131.2, 129.3, 128.6, 125.6, 124.6, 123.8, 122.5, 119.2, 117.0, 116.6, 110.0, 109.4, 108.7, 103.9, 82.8. MS(M-H): Calcd 459.0; Found 459.0.

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