Preparation of 5- and 6-Carboxyfluorescein

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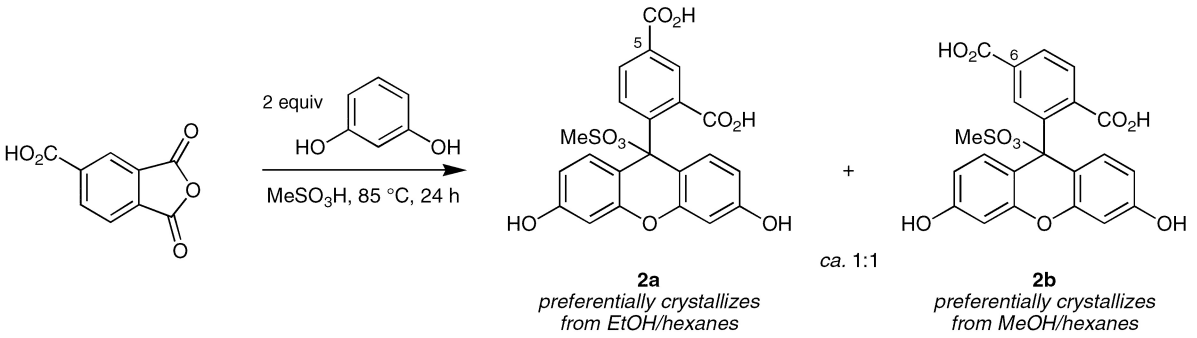
Abstract: Condensation of resorcinol with 4-carboxyphthalic anhydride in methanesulfonic acid gave a mixture of 5- and 6-carboxyfluorescein stereoisomers. These were separated by recrystallization from methanol– or ethanol–hexane to give 5- and 6-carboxyfluorescein, each in over 98% purity.

Key words: fluorescence, chromophores, condensation, heterocycles, regioselectivity



Scheme 1

Despite the widespread applications of 5- and 6-carboxyfluorescein **1**[1] as molecular labels,[2–4] it is surprisingly difficult to obtain these compounds as pure regioisomers. They can be purified from one another via preparative HPLC, and the price of commercial samples of these materials implies that this route is used in practice. Large-scale procedures for the preparation of these fluorescein derivatives would definitely be preferred.

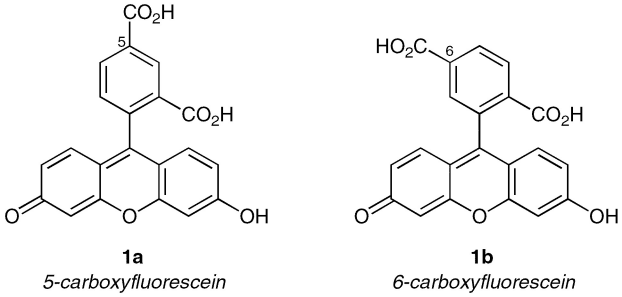


Figure 1

Regioisomeric fluorescein derivatives with polar substituents at the 5- and 6- positions can often be separated via fractional crystallization of lactone diester derivatives. This approach has been used for halogenated fluoresceins,[5–7] and it also has been reported for 5- and 6-carboxyfluorescein via a procedure that involves intermediates **A** (Figures 1 and 2).[8] However, others have claimed that the latter procedure is not easily reproduced,[3] and resorted to reduction of the 5- or 6-carboxylic acid functionality and separation of the regioisomers of this material instead. Reported here is a fractional crystallization procedure for the preparation of 5- and 6-carboxyfluorescein in multi-gram amounts. It has been reproduced several times in our laboratories and, unlike other routes to these and similar materials, it does not involve formation of diester lactone intermediates.

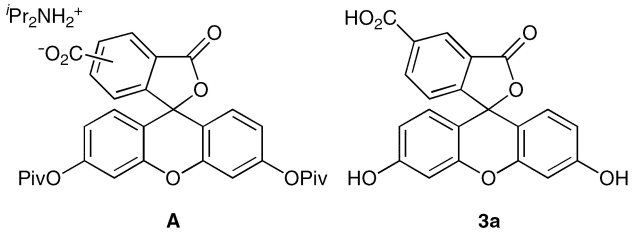


Figure 2

The key observation that led to the procedure reported here is that the material formed from condensation of 4-carboxyphthalic anhydride with 1,3-dihydroxybenzene in the presence of methanesulfonic acid was different to that

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formed when other acids were used (Scheme 1). Figure 3a shows the aromatic region of the ^{1}H NMR spectrum of the product **2a** formed from the condensation reaction, then purified via fractional crystallization (see below). Treatment of this with sodium hydroxide, then protonation with HCl gives a different material (Figure 3b); we propose this is the cyclic lactone **3a**. Conversely, product **2a** was regenerated when **3a** was treated with excess methanesulfonic acid. Assignment of structures **2a** and **2b** to the products of the initial reaction is supported by data from elemental analyses.

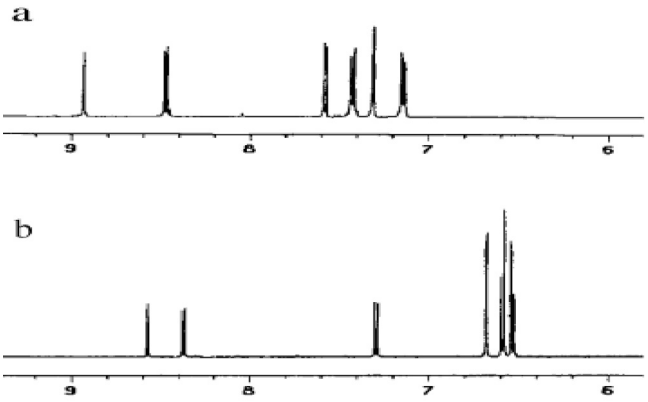

Figure 3 Aromatic ^{1}H NMR (CD_3OD) regions of: **a**, compound **2a**; and, **b**, compound **3a** formed from treatment of **2a** with NaOH then HCl. Differences in the chemical shifts were far less pronounced in NaOD–D_2O.

The reaction shown in Scheme 1 affords compounds **2a** and **2b** in approximately a 1:1 ratio. Recrystallization of 20 g of that mixture from methanol–hexane at –18 °C gave a crude sample of compound **2b**. A second recrystallization gave 1.0 g of this material in over 98% regioisomeric purity (Figure 4). Combination of the mother liquors, removal of the solvent, then two recrystallizations from a similar solvent system, ethanol–hexane, gave 3.2 g of the 5-carboxy isomer **2a** in over 98% purity. The mother liquors were again combined, the solvents were removed, and two recrystallizations of the residues from the original solvent system, methanol–hexane afforded another 3.0 g of the 6-isomer **2b**. Thus, in this particular experiment, the 5- and 6-isomers were isolated in 3.2 and 4.0 g amounts corresponding to 32 and 40% yields. No attempt was made to crystallize more material from the mother liquors, but it is likely that this is possible. In other experiments, compound **2a** was isolated first when the recrystallizing solvents were used in the reverse order (i.e. ethanol–hexane, then methanol–hexane).

The methanesulfonic esters **2** are easily converted to the 5- and 6-carboxyfluoresceins **1** by treatment with sodium hydroxide solution then neutralizing with aq HCl. Consequently, the procedures described here represent extremely convenient syntheses of the target materials **1a** and **1b** as highly enriched regioisomers.

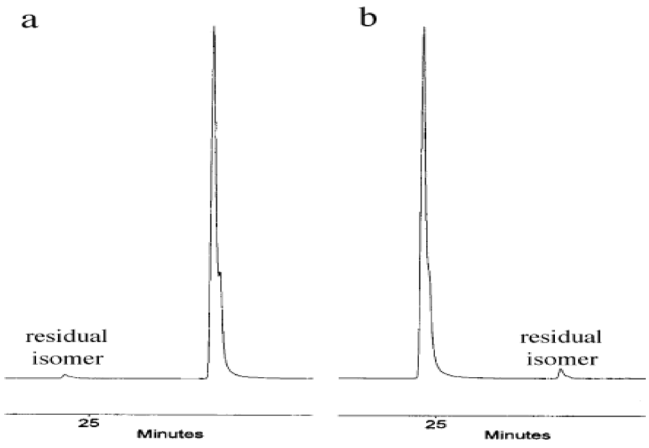

Figure 4 Analytical HPLC traces of: **a**, compound **2a** (98%); and, **b**, compound **2b** (98%) after two recrystallizations each (reverse phase C-18, Et_3N, HOAc, H_2O, MeCN).

High field NMR spectra were recorded on a Varian Unity Plus (^{1}H at 300 MHz, ^{13}C NMR at 75 MHz). Mass spectra were obtained from the Mass Spectrometry Laboratory at Texas A&M University. Resorcinol, 1,2,4-benzenetricarboxylic anhydride and methanesulfonic acid were purchased from Aldrich and used as received. Hexanes and MeOH were purchased from EMD Chemicals Inc. Absolute EtOH-200 proof was purchased from AAPER Alcohol. All solvents were used as received. Analytical HPLC were run on a SSI instrument (222C HPLC pump, 232C gradient controller) and a model 500 variable wavelength detector using a Supelco C-18 column (Supelcosil^{TM} LC-18-T, 25 × 4.6 mm, 5 μm), gradient elution was used (A = H_2O, B = MeCN, both with 0.1% v/v TEAA) with a constant flow rate of 0.9 mL/min.

5- and 6-Carboxyfluorescein (1a,b)

1,2,4-Benzenetricarboxylic anhydride (also called 4-carboxyphthalic anhydride, 25.0 g, 0.13 mol) was added to a solution of 1,3-dihydroxybenzene (also called resorcinol, 28.6 g, 0.26 mol) in methanesulfonic acid (1 M). An air condenser was attached to the flask and the reaction was heated at 85 °C in an open vessel for 24 h. After cooling to r.t., the reaction mixture was poured into 7 volumes of ice–H_2O. An orange–yellow precipitate formed; this was collected by filtration and dried in an oven at 200 ºC. This residue was recrystallized from MeOH–hexanes (2 ×) to give 6-carboxyfluorescein methanesulfonic acid adduct **2b** (1.0 g). The mother liquors from this procedure were collected, the solvent was removed in vacuo, and the residues were recrystallized from EtOH–hexanes (2 ×) to give 5-carboxyfluorescein methanesulfonic acid adduct **2a** (3.2 g). Finally, the mother liquors from this experiment were combined, evaporated to dryness, and recrystallized from MeOH–hexanes (2 ×) to give more of 6-carboxyfluorescein methanesulfonic acid adduct **2b** (another 3.0 g), making a total yield of 4.0 g (40%). Careful dropwise addition of concd aq HCl to solutions of these methanesulfonic esters **2a** and **2b** in aq NaOH (4 M) gave 5- (**1a**) and 6-carboxyfluorescein (**1b**), respectively, in near quantitative yield.

Compound 1a

Mp 385–388 °C (lit[8] 368–372 °C).

^{1}H NMR (300 MHz, NaOD–D_2O): δ = 6.56 (d, *J* = 2.47 Hz, 2 H), 6.66 (dd, *J* = 2.21, 9.36 Hz, 2 H), 7.20 (d, *J* = 9.08 Hz, 2 H), 7.33 (d, *J* = 7.71 Hz, 1 H), 8.07 (dd, *J* = 1.65, 7.84 Hz, 1 H), 8.26 (d, *J* = 1.93 Hz, 1 H).

^{13}C NMR (75 MHz, NaOD–D_2O): δ = 103.4, 114.2, 121.7, 128.8, 129.9, 130.1, 131.7, 133.9, 137.8, 139.7, 158.0, 158.5, 174.55, 174.57, 176.4.

MS (ESI-TOF): *m*/*z* = 375 $(M - H)^-$.

Anal. Calcd for $C_{21}H_{12}O_7 \cdot 1.5H_2O$: C, 62.53; H, 3.75. Found: C, 62.70; H, 3.49.

Compound 1b
Mp 372–374 °C (lit[8] 352–356 °C).

^{1}H NMR (300 MHz, NaOD–D_2O): δ = 6.68 (m, 4 H), 7.24 (d, *J* = 9.35 Hz, 2 H), 7.86 (d, *J* = 1.1 Hz, 1 H), 7.89 (s, 1 H), 8.09 (dd, *J* = 1.51, 8.11 Hz, 1 H).

^{13}C NMR (75 MHz, NaOD–D_2O): δ = 103.3, 115.0, 121.3, 128.6, 130.2, 130.4, 131.1, 131.9, 137.5, 141.7, 157.9, 158.2, 174.3, 174.6, 175.3.

MS (ESI-TOF): *m*/*z* = 375 $(M - H)^-$.

Anal. Calcd for $C_{21}H_{12}O_7 \cdot 1.5H_2O$: C, 62.53; H, 3.75. Found: C, 62.43; H, 3.36.

Compound 2a
Mp 196–198 °C.

^{1}H NMR (300 MHz, NaOD–D_2O): δ = 2.80 (s, 3 H), 6.53 (d, *J* = 2.10 Hz, 2 H), 6.60 (dd, *J* = 2.40, 9.60 Hz, 2 H), 7.13 (d, *J* = 9.30 Hz, 2 H), 7.21 (d, *J* = 7.80 Hz, 1 H), 8.02 (dd, *J* = 1.80, 9.00 Hz, 1 H), 8.23 (d, *J* = 1.20 Hz, 1 H).

^{13}C NMR (75 MHz, NaOD–D_2O): δ = 38.57, 103.8, 112.3, 123.1, 128.6, 129.6, 130.2, 131.5, 134.2, 137.5, 139.8, 158.7, 158.8, 174.7, 174.9, 180.7.

MS (ESI-TOF): *m*/*z* = 471 $(M - H)^-$.

Anal. Calcd for $C_{22}H_{16}O_{10}S \cdot H_2O$: C, 53.88; H, 3.70; S, 6.54. Found: C, 53.66; H, 3.86; S, 6.61.

Compound 2b
Mp 318–321 °C.

^{1}H NMR (300 MHz, NaOD–D_2O): δ = 2.79 (s, 3 H), 6.61 (m, 4 H), 7.19 (d, *J* = 9.60 Hz, 2 H), 7.76 (s, 1 H), 7.84 (d, *J* = 8.10 Hz, 1 H), 8.06 (dd, *J* = 1.20, 9.00 Hz, 1 H).

^{13}C NMR (75 MHz, NaOD–D_2O): δ = 38.58, 103.5, 114.2, 121.9, 128.5, 130.1, 130.5, 131.2, 131.9, 137.5, 141.9, 158.3, 158.5, 174.4, 174.8, 177.2.

MS (ESI-TOF): *m*/*z* = 471 $(M - H)^-$.

Anal. Calcd for $C_{22}H_{16}O_{10}S \cdot 2H_2O$: C, 51.97; H, 3.96; S, 6.31. Found: C, 52.30; H, 4.02; S, 6.23.

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