Preparation of 5- and 6-Carboxyfluorescein

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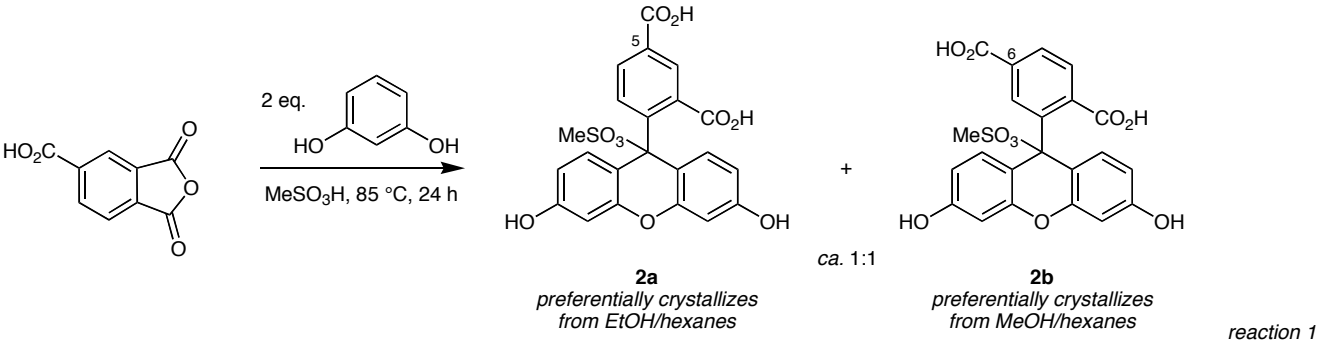
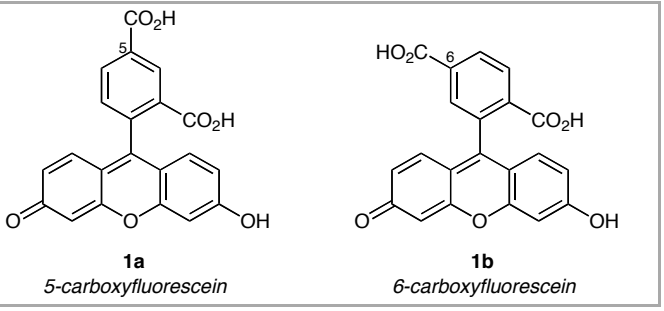
Abstract: Condensation of resorcinol with 4-carboxyphthalic anhydride in methane sulfonic acid gave a mixture of 5- and 6-carboxyfluorescein stereoisomers. These were separated by recrystallization from methanol- or ethanol-hexane to give 5- and 6-carboxyfluorescein, each in over 98 % purity.

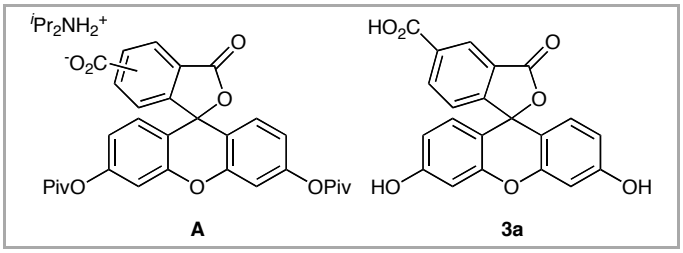
Key words: fluorescence, chromophores, condensation, heterocycles, regioselectivity



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.



Regioisomeric fluorescein derivatives with polar substituents at the 5- and 6- positions can often be separated via fractional cyclization 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 involve intermediates **A**.[8] However, others have claimed that the latter procedure is not easily reproduced,[3] and resorted to reduction of the 3-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 multigram 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.



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

2b to the products of the initial reaction is supported by data from elemental analyses.

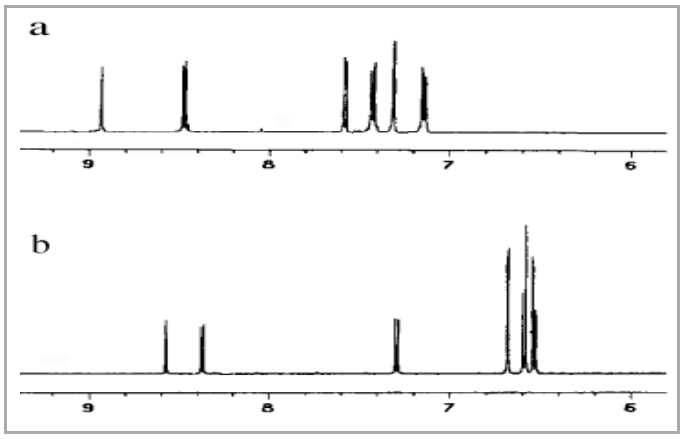


Figure 1. Aromatic ^{1}H NMR (CD_3OD) regions of: **a**, compound **2a**; and, **b**, compound **3** formed from treatment of **2a** with NaOH then HCl. Differences in the chemical shifts were far less pronounced in $NaOD/D_2O$.

Reaction 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 2). 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 g 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 (*ie* ethanol/hexane then methanol/hexane).

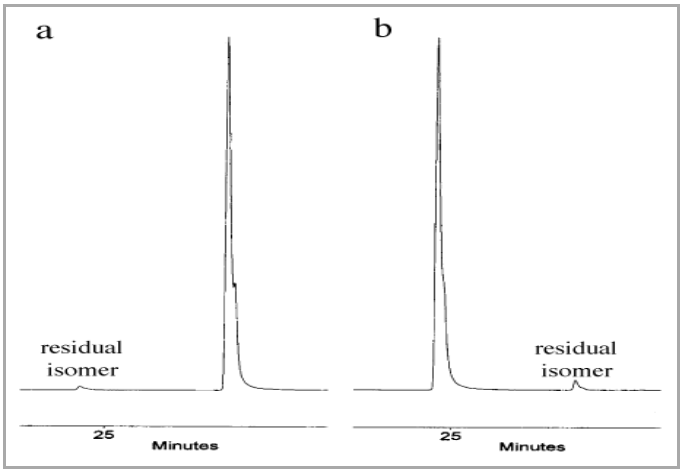


Figure 2. Analytical HPLC traces of: **a**, compound **2a** (98 %); and, **b**, compound **2b** (98 %) after two recrystallizations each. (reverse phase C-18, NEt_3, HOAc, H_2O, MeCN).

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

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

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 methane sulfonic acid (1M). An air condenser was attached to the flask and the reaction was heated at 85 ºC in an open vessel for 24 h. After cooling to room temperature, the reaction mixture was poured into 7 volumes of ice/water. An orange-yellow precipitate formed; this was collected by filtration and dried in an oven at 200 ºC. This residue was recrystallized two times from methanol/hexane to give 1.0 g of 6-carboxyfluorescein methanesulfonic acid adduct **2b**. The mother liquors from this procedure were collected, the solvent was removed *in vacuo*, and the residues were recrystallized two times from ethanol/hexanes to give 3.2 g of 5-carboxyfluorescein methanesulfonic acid adduct **2a**. Finally, the mother liquors from this experiment were combined, evaporated to dryness, and recrystallized two times from methanol/hexanes to give another 3.0 g of 6-carboxyfluorescein methanesulfonic acid adduct **2b**, making a total yield of 4.0 g, 40 %. Careful dropwise addition of conc. $HCl_{(aq)}$ to solutions of these methanesulfonic esters **2a** and **2b** in 4M sodium hydroxide gave 5- (**1a**) and 6-carboxyfluorescein (**1b**), respectively in near quantitative yield.

1a: ^{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 $C_{21}H_{12}O_7$ + 1.5 H_2O: C, 62.53; H, 3.75. Found: C, 62.70; H, 3.49.

Mp 385-388 ºC. Lit[8] 368 – 372 °C.

1b: ^{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, 1H), 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 $C_{21}H_{12}O_7$ + 1.5 H_2O: C, 62.53; H, 3.75. Found: C, 62.43; H, 3.36.

Mp 372-374 ºC. Lit[8] 352 – 356 °C.

2a: ^{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 $C_{22}H_{16}O_{10}S$ + H_2O: C, 53.88; H, 3.70; S, 6.54. Found: C, 53.66; H, 3.86; S, 6.61.

Mp 196-198 ºC.

2b: ^{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, 1H), 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 $C_{22}H_{16}O_{10}S$ + 2 H_2O: C, 51.97; H, 3.96; S, 6.31. Found: C, 52.30; H, 4.02; S, 6.23.

Mp 318-321 ºC.

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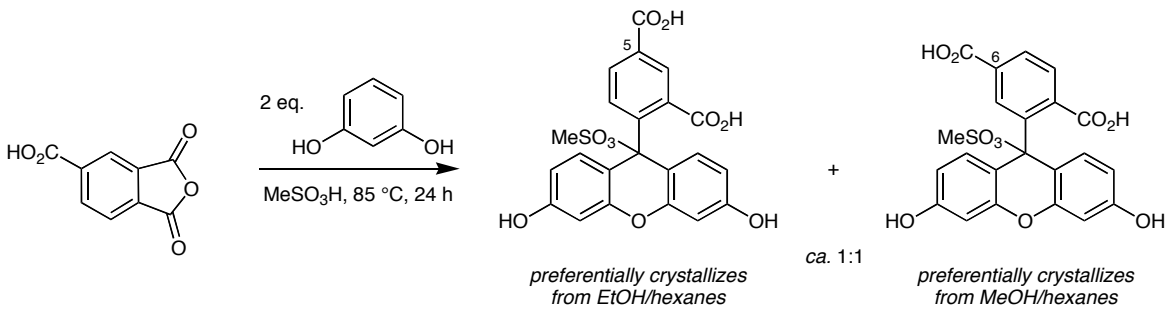
graphical abstract



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