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Improved Synthetic Procedures for 4,7,2′,7′-Tetrachloro- and 4′,5′-Dichloro-2′,7′-dimethoxy-5(and 6)-carboxyfluoresceins

Matthew H. Lyttle,* Tim G. Carter, and Ronald M. Cook

Biosearch Technologies, Inc., 81 Digital Drive, Novato, California 94949, U.S.A.

Abstract:

Literature syntheses of 4,7,2′,7′-tetrachloro-5(and 6)-carboxyfluorescein ("5 and 6 TET") 1 and 4′,5′-dichloro-2′,7′-dimethoxy-5(and 6)-carboxyfluorescein ("5 and 6 JOE") 2 are reviewed, and new, preparatively useful methods are presented. A three-step synthesis of 1 was developed, which proved to be more efficient than the published seven step synthesis of this compound. The published synthesis of 2 also proved difficult to reproduce, and a better workup of the key intermediate 2-chloro-4-methoxy resorcinol was devised. Isolation of purified single isomers of both dyes is described.

Introduction

The carboxy-functionalized fluorescein[1] and rhodamine[2] dyes have become increasingly important as conjugated fluorescent markers of biologically active compounds. Although these dyes share a common, xanthene-based skeleton, different substituents can be made to cause marked differences in absorbance and fluorescence emission wavelengths. The similar structure of the compounds facilitates parallel synthesis of different tagged DNA or protein molecules with discrete absorbance and emission wavelengths. These properties have been widely exploited in DNA sequencing[3] and other applications. Selective substitution of chlorine for aromatic hydrogen has also been seen to increase fluorescence efficiency and to narrow emission and absorbance maxima (vs λ) compared with fluorescein.[4] These characteristics are important when analyzing mixtures of fluorescently tagged molecules.

Carboxy-functionalized fluorescein dyes first appeared in the literature many years ago,[5] but there is very little modern peer-reviewed information regarding synthesis and purification of these important compounds. Recent related publications include reduction of carboxy-functionalized fluoresceins and conjugation of the resulting hydroxymethyl fluorescein derivatives to DNA[6] and a paper on the synthesis of fluorinated fluoresceins.[7] An online data search on **1** and **2** led to patents published by Khanna, et al. in 1982.[8,9] There is also a later patent[4] describing the compounds, with reference to the Khanna patent for synthetic intermediates. For each synthesis there is no reference to the starting materials, 3-nitro-2,5-dichloro-*p*-xylene for **1** and 2-chloro-3-hydroxy-4-methoxy benzaldehyde for **2**. The compounds are not commercially available, however, preparations of both of the materials were found in the older literature.[10,11]

This report reviews the synthesis of **1** and **2** using the patent literature procedures,[4,8,9] which provide small amounts of the materials through intermediates which are sometimes not well-characterized. We describe our attempts to reproduce these methods and then establish alternative procedures which provide more practical and reliable production of multigram quantities of these important dyes.

Results and Discussion

Synthesis of 1. First, the literature synthesis[8] of 3,6-dichlorotrimellitic acid **5** was reviewed. See Figure 1. The synthesis involves six steps starting with commercially available 2,5-dichloro-*p*-xylene **3**. Aromatic nitration of **3** followed by reduction gave 2,5-dichloro-3,6-dimethyl aniline. Conversion to nitrile 2,5-dichloro-3-cyano-*p*-xylene was accomplished via diazotization followed by cyanide addition. The resulting nitrile was then hydrolyzed in two steps to 2,5-dichloro-3,6-dimethylbenzoic acid, and then the remaining benzylic methyl groups were oxidized with $KMnO_4$ to give 2,5-dichloro-1,3,4-trimellitic acid **5**. Heating **5** with 4-chlororesorcinol and $ZnBr_2$ gave **1**. No final calculation of yield of product was given in any of the references.[4,8,9]

We began by nitration of **3** with fuming HNO_3 in H_2SO_4 at 60 °C, which gave 3-nitro-2,5-dichloro-*p*-xylene in good yield.[10] Reduction to the amine was then tried by both $SnCl_2$ reduction and catalytic hydrogenation. Both methods gave low yields of impure products. At this point an alternate scheme was devised (see Figure 2). 2,5-dichloro-*p*-xylene **3** was treated with acetyl chloride and aluminum trichloride in refluxing dichloroethane for 5 h. The Friedels−Craft adduct, 2,5-dichloro-3-acyl-*p*-xylene **4** was obtained in 60% yield as a beige solid on simple extraction. This material was then treated with alkaline $KMnO_4$ in boiling water to give **5** in 44% yield as a white solid. Heating with 4-chlororesorcinol in methane sulfonic acid[4] gave **1**. Preparatively useful 20−30 g batches of **1** were routinely produced with the new method, far more efficiently than (our attempts with) the literature method. The overall yield was

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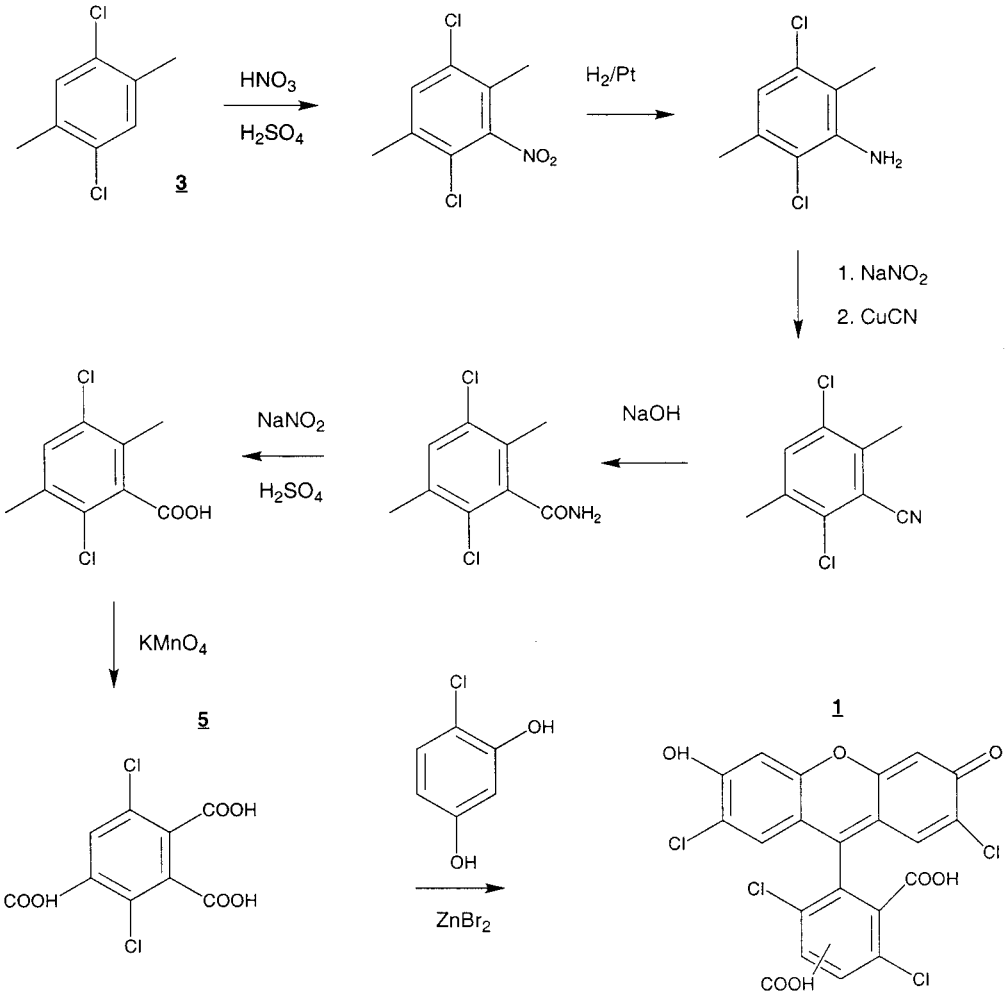

Figure 1. **Literature Synthesis of 1.**

21%. Single isomer 6-carboxy **1** was then prepared by adapting the methods developed to separate 5- and 6-carboxy fluorescein;[1] this method also provided pure 6-carboxy isomer material protected as its bis-pivaloylate which could be further elaborated into protected conjugates.

Synthesis of 2. Here, the literature preparation[8] was more faithfully followed, once the starting material was synthesized. See Figure 3. As per Faulkner and Woodcock,[11] isovanillin (4-methoxy-3-hydroxy benzaldehyde) **6** was chlorinated by bubbling Cl_2 through a solution of the aldehyde in $CHCl_3$. The product, 4-methoxy-3-hydroxy-2-chloro benzaldehyde **7** was isolated by simple filtration in 90% yield. NMR showed an AB pattern for the two aromatic protons, consistent with chlorine addition at the 2-position. Recrystallized material had almost the same melting point as that given in the literature.[11] Next, a Baeyer−Villiger oxidation with MCPBA of the aldehyde to the corresponding formate ester **8** was undertaken. Normally, aldehydes do not undergo this type of reaction and are simply oxidized to the corresponding carboxylic acid. The special case of Baeyer−Villiger-type reaction in aldehyde-functionalized phenols was first demonstrated by Dakin[12] and appears to operate in this instance. Electron density from a phenolic or anisolic oxygen ortho or para to the benzylic aldehyde most likely contributes to formation of an oxirane intermediate during peracid oxidation, which opens to the observed product formate ester. After oxidation, alkaline hydrolysis of the ester provides the desired phenol, 2-chloro-4-methoxy resorcinol **9**. Workup and isolation of the product proved to be quite difficult, since **9** is somewhat water soluble. The literature procedure[8] produced a black oil from which no crystals could be obtained, and no **2** was obtained in the subsequent reaction with the material. The acid byproduct from the MCPBA oxidation, 2-chlorobenzoic acid, is very hard to remove from the product and interferes with subsequent chemistry. We modified the workup of the MCPBA reaction so that most of the 2-chlorobenzoic acid is removed before hydrolysis of the formate ester **8** to the desired product **9**. Even then, crystallization of **9** was inconsistent, and column chromatography was sometimes needed to obtain pure material. Our crystalline **9** also was obtained as dense cubic crystals, not white needles as reported.[8] **9** reacted with trimellitic anhydride in methane sulfonic acid to give **2**. Multiple gram batches of **2** were consistently produced with this method. Our overall yield of **2** was 8%, and no overall yield could be calculated for the literature method.[8]

Purification and Analysis of 1 and 2. Both **1** and **2** were obtained as red solids after reaction, precipitated by dropwise

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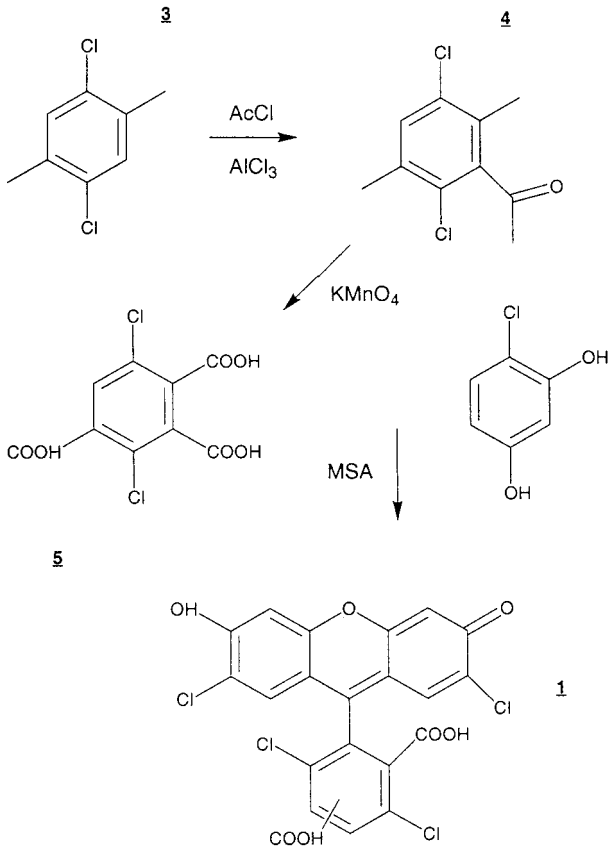

Figure 2. **Improved Synthesis of 1.**

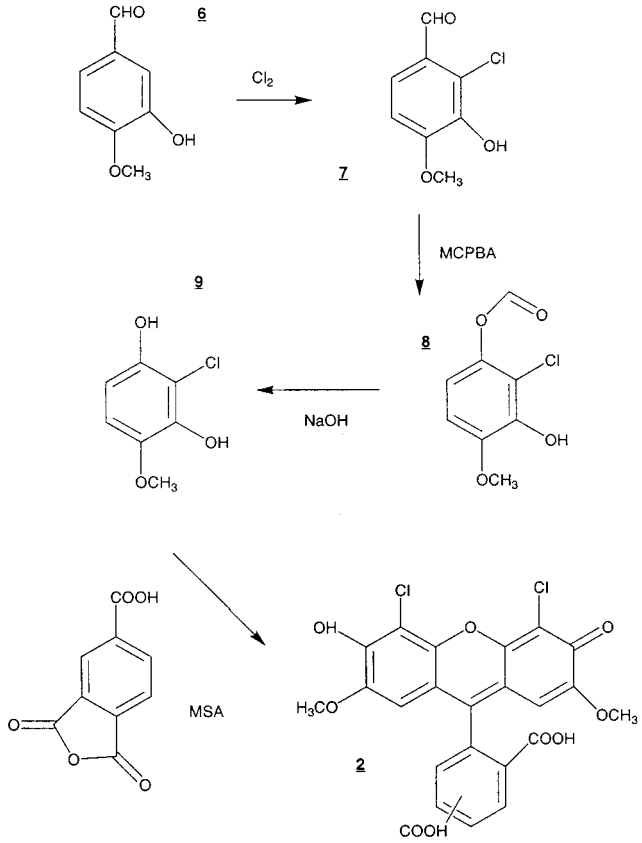

Figure 3. **Synthesis of 2.**

addition of the methane sulfonic acid reaction mixtures to water. The materials were dried for several days under vacuum. Reverse phase HPLC of the materials showed both 5- and 6-carboxy isomers present. The crude **1** reaction product was fairly clean, 74% of the total peak area due to combined dye isomers at 254 nm, while the **2** crude product was only about 55% (5- and 6-carboxy) **2**. See Figures 4 and 5. Attempts to generate isomerically pure **1** using the literature procedure[4] involving conversion to the diacetate and crystallization did not work in our hands, and the chromatographic conditions[8] outlined in Khanna, et al. for both **1** and **2** were also ineffective (silica, 10% MeOH/DCM with 1% HOAc). We found that the materials could be purified by open column chromatography on neutral alumina eluted with an aqueous ammonia−2-propanol mix (Complete details in the Experimental Section). The major colored components were collected: the **1** purified product was now >90% pure (as a ~2:3 mixture of isomers, data not shown). For **2**, >95% pure single isomer dye was obtained, the identity was confirmed as the 6-carboxy isomer by comparison to a standard.[13] A later fraction contained a small amount of the 6-isomer as well as mostly the 5-isomer as a 2:7 mixture. See Figure 5. Absorption and emission spectra of these purified samples of **1** and **2** agreed with the literature values.[14,15] **1** was converted into its bis-pivaloylate with methods analogous to those published for separating 5- and

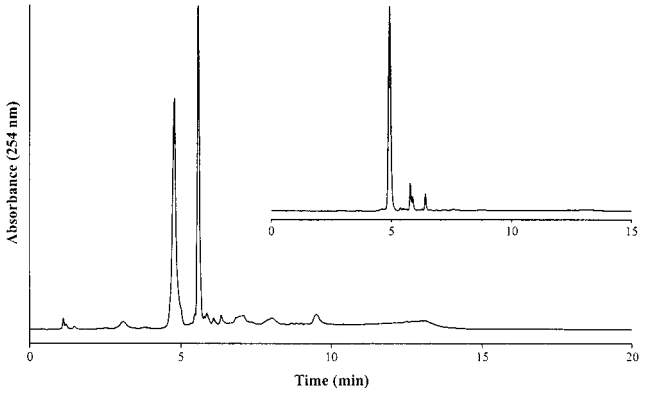

Figure 4. **Reverse phase HPLC of crude 1. Inset shows pure 6-isomer of 1 purified by crystalizing bis-pivaloylate derivative.**

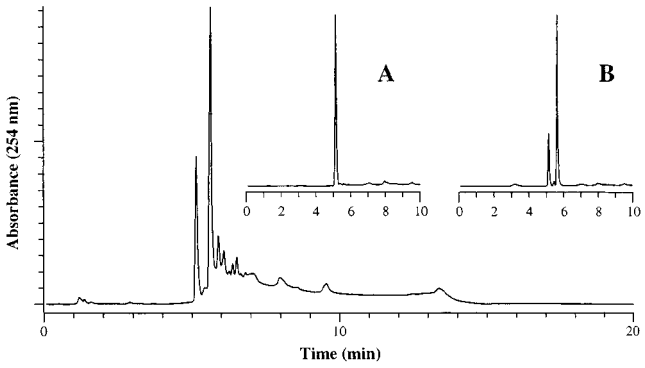

Figure 5. **Reverse phase HPLC of crude 2. Inset A shows pure 6-isomer of 2 purified by column chromatography. Inset B shows later fraction containing mostly 5-isomer.**

(13) Obtained from Molecular Probes, Eugene, Oregon.
(14) Rudert, W. A.; Braun, E. R.; Faas, S. J.; Menon, R.; Jaquins-Gerstl, A.; Trucco, M. *Biotechniques* **1997**, *22*, 1140.
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6-carboxy fluorescein,[1] and removal of the pivaloyl groups with base followed by precipitation with aqueous acid gave material which contained >90% pure 6-carboxy **1**. See Figure 4 (inset).

Conclusions

Improved methods have been applied to the synthesis and purification of two chlorinated fluoresceins. We hope that these updated procedures will make **1** and **2**, as well as similar compounds, more accessible to those workers who require them.

Experimental Section

Aqueous ammonia, sulfuric acid, acetic acid, and concentrated HCl were reagent grade from J.T. Baker. Ethyl ether, 1,2-dichloroethane, dichloromethane, chloroform, ethyl acetate, 2-propanol, toluene, acetonitrile, ethanol, methanol, and petroleum ether were Omnisolve grade from VWR. Aluminum trichloride, 2,5-dichloro-*p*-xylene, acetyl chloride, potassium permanganate, oxalic acid, isovanillin, chlorine gas, *m*-chloroperoxybenzoic acid (MCPBA), sodium hydroxide, potassium carbonate, sodium bicarbonate, magnesium sulfate, 4-chlororesorcinol, trimellitic acid anhydride, chromatographic alumina, and methane sulfonic acid were from Aldrich. Chromatographic silica was from Grace Chemical Co. Elemental analyses were performed by Desert Analytics (Tuscon, AZ), and NMR work was done by Acorn NMR (Fremont, CA). MALDI Mass spectra were done in-house on a Finnigan Lasermat.

Synthesis of 1. *3-Acyl-2,5-dichloro-p-xylene **4***. In a 3 L round-bottom flask with reflux condenser and magnetic stirring under argon was added 2,5-dichloro-*p*-xylene (100 g, 0.571 mol) dissolved in 1400 mL of ethylene dichloride, along with acetyl chloride (55 mL, 0.77 mol). The mixture was heated to reflux, and aluminum trichloride (103 g, 0.77 mol) was added as a solid in small portions over 1.5 h. Reflux was continued another 4 h. The large volume of HCl gas which evolved was allowed to pass out of the flask into a fume hood. The solution was cooled, and solids were removed by filtration. The organic phase was poured into 1 L of cracked ice, and after 20 min 200 mL of concentrated aqueous ammonia was added. The mixture was placed in a 4 L separatory funnel along with 400 mL of 2 M HCl, 600 mL of CH_2Cl_2, and 500 mL of water. A rod was used to gently stir the aqueous layer. (An emulsion may form if the separatory funnel is shaken in a normal manner.) The layers were allowed to separate overnight. The organic phase was dried over $MgSO_4$ and evaporated to give 75 g (60% yield) of a beige solid. A small amount of the product was purified by column chromatography on silica; a gradient of 0−20% CH_2Cl_2 in pet ether over 3 L of mobile phase was used to obtain pure **4** as tan crystals, $R_f = 0.8$ in 2% MeOH/CH_2Cl_2, mp 97−101 °C. H^1 NMR ($CDCl_3$, δ): 7.25 (s, 1 H), 2.5 (s, 3H), 2.3 (s, 3H), 2.1 (s, 3H).

Anal. Calcd for $C_{10}H_{10}OCl_2$: C, 55.33; H, 4.68. Found: C, 56.00; H, 4.75.

2,5-dicholoro-1,3,4-trimellitic acid, **5**.

Crude **4**, 25 g (0.11 mol) was dissolved in a solution of 500 mL of water, 50 g of K_2CO_3, and 90 g of (0.57 mol) $KMnO_4$. The purple solution was refluxed for 4 h and allowed to cool and stand overnight. The solution was acidified to pH 1 with 6 N H_2SO_4, and oxalic acid was added until the solution became colorless. The mixture was filtered and the aqueous phase extracted with 1000 mL of ether, and the extract was then dried over $MgSO_4$ and evaporated to give 14 g (44% yield) of **4** as a white solid, mp 226−230 °C (lit[8] 232−233 °C). ^{1}H NMR (d_6-DMSO, δ): 14−12 (broad s, 3H), 8, (s, 1H).

Anal. Calcd for $C_9H_4O_6Cl_2 \cdot 1/3H_2O$: C, 37.92; H, 1.64. Found: C, 38.05; H, 1.68.

5(and 6)-Carboxy-4,7,2′,7′-tetrachlorofluorescein ("5 and 6 TET"), 1. A tared 500 mL round-bottom flask containing 11.4 g (41 mmol) of **5** was heated to 150° for 20 min in a fume hood. Water was observed to pass out of the flask. The flask was sparged briefly with argon and allowed to cool. The flask was re-weighed, and contained 10.5 g of now dark solid. 4-Chlororesorcinol, 10.5 g (7.4 mmol), was added, along with 100 mL of methane sulfonic acid. The mixture was heated for 2 h at 140−150°. The dark red mixture was cooled and poured slowly into 1500 mL of rapidly stirred water. The brown−yellow solid was collected by suction filtration and washed with 300 mL of water, and air-dried for several days. The yield was 15.5 g (79%). Reverse Phase HPLC with photodiode array detection of the crude product showed 74% of the total peak area due to combined dye isomers at 254 nm. MALDI MS showed MH^+/e 512.86 amu, Calcd 512.910 amu.

6-Carboxy-4,7,2′,7′-tetrachlorofluorescein Dipivalate Diisopropylamine Salt. 1, 20 g (40 mmol), was dissolved in 200 mL of dry pyridine and reduced to a tar by rotary evaporation. Another 200 mL of dry pyridine was added, and the solution was chilled to 0° C in an ice bath under argon. Pivaloyl chloride (30 mL, 29 g, 244 mmol) was then added dropwise over 30 min, and the black mixture was allowed to stand and warm to room temperature overnight. Water, 20 mL, was added dropwise over 10 min with stirring, and the solution was evaporated to a tar by rotary evaporation. The dark residue was dissolved in 400 mL of EtOAc and washed successively with 300 mL portions of of 1 M HCl, saturated $NaHCO_3$, and then 1 M HCl. The organic phase was dried over $MgSO_4$ and evaporated and then redissolved in 250 mL of anhydrous ethanol. Diisopropylamine, 30 mL, was added, and the mixture was chilled at −20° C for 2 days. A salmon colored precipitate was collected by filtration on a medium porosity glass frit and the solid washed with a small amount of cold ethanol. Vacuum drying gave 8 g of solid. The mother liquor was concentrated to 150 mL and re-chilled, affording another 2.5 g of the same solid. The combined yield was 10.5 g, Both crops were analytically pure.

Anal. Calcd for $C_{37}H_{39}NO_9Cl_4$: C, 56.75; H, 5.01; N, 1.79.

Crop 1, Found: C, 56.79; H, 5.06; N,1.71.

Crop 2, Found: C, 56.69; H, 5.11; N,1.91.

6-Carboxy-4,7,2′,7′-tetrachlorofluorescein. 2′,7′-Dichloro-6-carboxy-4,7-dichlorofluorescein dipivalate diisopropylamine salt, 100 mg, was treated with a mixture of 3 mL of concentrated aqueous ammonia and 3 mL of methanol for several days. The bright yellow solution was filtered and allowed to evaporate for several days. HCl (2 M, 5 mL) was added, and the orange suspension was chilled to 0° C overnight. The yellow solid was collected and dried under vacuum to give 55 mg (84% yield) of 6-carboxy **1**.

Anal. Calcd for $C_{21}H_8O_7Cl_4 \cdot 3/2\ H_2O$: C, 46.61; H, 2.04. Found: C, 46.82; H, 1.94.

Synthesis of 2. *2-Chloro-4-methoxy Resorcinol, 9.* 4-methoxy-3-hydroxy benzaldehyde, 100 g (0.66 mol) was dissolved in 1200 mL of $CHCl_3$ with gentle heating and stirring. Chlorine gas was slowly bubbled into the solution for 2 h, whereupon a thick precipitate formed. The mixture was allowed to cool and the solid collected by filtration. The yield was 106 g (86%) of a white solid, mp 196−200 °C. A small amount of the product was recrystallized from MeOH−H_2O to give white crystals, mp 203−205 °C (lit.[11] mp 205−206 °C). The solid (100 g, 0.53 mol) was mixed with 160 g (0.71 mol) of 77 wt % MCPBA in 1200 mL of CH_2Cl_2 and refluxed 4 h. The solution was cooled overnight, and solids were removed by filtration. The solid cake was washed with an additional 500 mL of CH_2Cl_2 and the combined organic liquid washed with two 800 mL portions of saturated aqueous $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated to a solid. The solid was treated with 300 mL of 0.5 N NaOH overnight, and became very dark. The solution was adjusted to pH 1 with concentrated HCl, and solids were removed by filtration. The dark aqueous solution was washed twice with 500 mL of ether, and the combined organic phases evaporated to a black oil after drying over $MgSO_4$ and filtration. The oil was purified by chromatography on a bed of 600 mL of silica packed in CH_2Cl_2 (1% pyridine) with a gradient to 2% MeOH over 3 L of mobile phase. Pure fractions, $R_f = 0.6$, 2% MeOH/CH_2Cl_2, were pooled, washed with dilute HCl, and stripped to provide 19.5 g (22% yield) of **9** as a pink wax. Crystalline material could be obtained by dissolution in EtOAc, addition of pet ether to a cloud point, and chilling, mp 74−76 °C (lit.[8] 69−70 °C). ^{1}H NMR ($CDCl_3$, δ): 6.7(d, $J = 9$ Hz, 1H), 6.5(d, $J = 9$ Hz, 1H), 6.0 (broad s, 1H), 5.3 (broad s, 1H), 4.0 (s, 3H).

Anal. Calcd for $C_7H_7O_3Cl$: C, 48.16; H, 4.04. Found: C, 47.87; H, 3.74.

4′,5′-Dichloro-2′,7′-dimethoxy-5(and 6)-carboxyfluorescein ("5 and 6 JOE") 2. 9, 19.5 g (0.11 mol) and 8 g (42 mmol) of trimellitic acid anhydride were added to 40 mL of methane sulfonic acid. The mixture was stirred and heated to 160° C for 30 min and cooled to room temp. The dark mixture was poured into 500 mL of rapidly stirred water, and the solid material collected by filtration through a sintered glass funnel. The material was dried for several days under vacuum to give 9 g (42% yield) of **2** as a brick red solid. A column 5 × 10 cm of neutral alumina, pretreated with 7 wt % water, was prepared with a 30% (28% aqueous) ammonia and 70% 2-propanol mobile phase. Crude **2** (900 mg) was dissolved in 50 mL of the above mobile phase and applied to the column. The product was collected as the second and third major colored band which eluted from the column. The solutions were stripped and dried for several days under vacuum. Pure 6-carboxy isomer **2** (240 mg) was obtained, as well as 180 mg of mostly 5-carboxy isomer **2** which contained some 6-carboxy **2** isomer. MALDI MS showed *m*/*e* 528.07 amu (MNa^+), Calcd 527.0 amu. An analytical sample was prepared by dissolving 20 mg of pure 6-isomer **2** in 3 mL of methanol containing 0.5 mL of concentrated ammonia, precipitating the dye with 2 N HCl, collecting the solid after overnight chilling and high vacuum for several days.

Anal. Calcd (for pure 6-carboxy **2**) $C_{23}H_{14}O_9Cl_2 \cdot HCl \cdot 2H_2O$: C, 47.81; H, 3.31. Found: C, 48.07; H,3.17.

Analytical HPLC Conditions. The dye (1−2 mg) was dissolved in 10 mL of ethanol, and two drops of concentrated ammonium hydroxide was added. The solution (1 mL) was diluted with 9 mL of water, and 10 μL of this solution was injected onto a Ranin Microsorb 12 cm × 5 mm C-18 column on a Waters Millenium HPLC system. The column was eluted with a gradient of 100% A (0.1 N ammonium acetate) for 2 min, 100% A to 90% A (B was 100% CH_3CN) over 10 min, then 90% A to 100% B over 10 min, then back to 100% A over 1 min. The flow rate was 1 mL/min, with absorbance read at 254 nm.

Fluorescence Emission and Absorbance Analysis. Absorbance maxima were read by photodiode array detector on the HPLC system above. Both 5- and 6-carboxy **1** had λ_{max} of 521.4 nm. The 6-carboxy **2** had λ_{max} of 523.8,[14] and The 5-carboxy **2** had λ_{max} of 526.2.[15] For emission maxima, the same ethanolic solutions above were diluted with water and read in a Gemini Spectra-Max (Molecular Devices). The mix of 5- and 6-carboxy **1** had λ_{max} (emission) at 540 nm,[14] excitation at 510 nm, and the pure 6-carboxy **2** had λ_{max} (emission) of 550,[15] excitation at 510 nm.

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