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Mild Synthesis of Asymmetric 2′-Carboxyethyl-Substituted Fluoresceins

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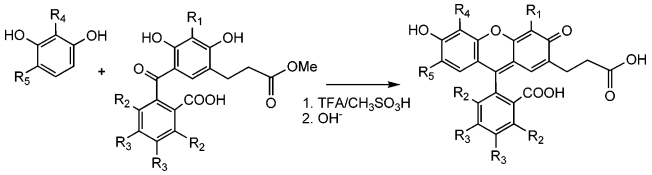
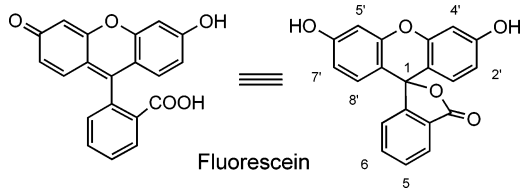


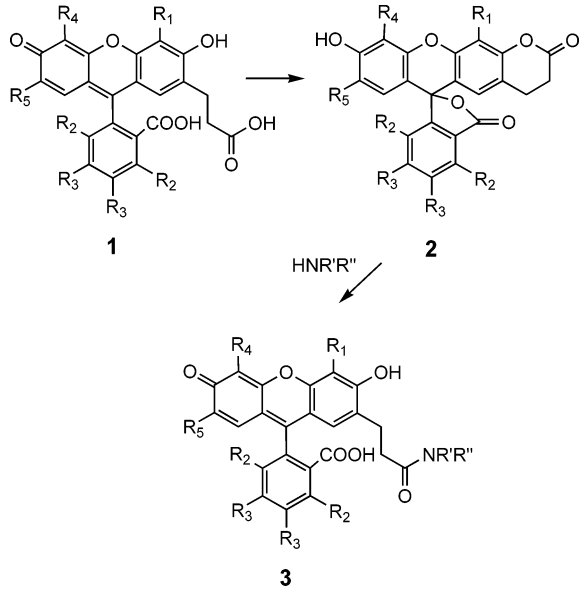
Asymmetric fluoresceins bearing a carboxyethyl group in the chromophoric portion of the dyes were prepared by a reaction of substituted phthalic anhydride with a carboxyethyl substituted resorcinol analogue followed by a condensation with a second resorcinol analogue. In order to avoid an accumulation of symmetric side products, the second step was performed in two substeps: acid-catalyzed formation of a triphenylmethyl intermediate followed by base-catalyzed cyclization which furnished the desired dyes.

Fluoresceins bearing tethered functional groups are used in the preparation of fluorescent conjugates as well as intermediates for more elaborate reagents such as dye phosphoramidites.[1a−e] The most commonly used activated fluoresceins are derived from their amino- or carboxy-substituted analogues with the substituents located in the carboxyphenyl portion of the dyes.



These compounds are typically prepared as mixtures of 5- and 6-isomers by a reaction of 4-substituted phthalic anhydrides (such as 1,2,4-benzenetricarboxylic anhydride) with appropriate resorcinol analogues in the presence of strong acids or $ZnCl_2$. The isomers can be separated by fractional crystallization as reported for 5(6)-carboxyfluorescein,[2a,b] 4,7,2′,7′-tetrachloro-5(6)-carboxyfluorescein,[3] fluorescein-5(6)-sulfonic acid,[4] 5(6)-bromofluorescein,[5] and 2′,7′-dichloro-5(6)-carboxyfluorescein.[6] In most cases dye mixtures should be transformed into the bis-acylated lactone form before crystallization. As a result, this procedure requires additional protection/deprotection steps and is not universally applicable.

SCHEME 1. Preparation of Fluorescent Conjugates from 2′-Carboxyethyl-Substitited Fluoresceins



An alternative strategy for the preparation of functionalized fluoresceins is based on a modification of the xanthenone fragment of the dyes. Asymmetric 2′-carboxyethyl-substituted rhodol dyes[7,8] are examples of this approach. Mono-2′-carboxyethyl substituted fluoresceins of structure **1** have also been suggested.[9] However, to our knowledge, no actual compounds have been prepared. A useful attribute of these analogues is their ability to form intramolecular fused lactones **2**. Given that the pK_a of the phenolic group for most fluorescein analogues is below 7, the activity of the lactones should be suitable for selective reaction with amine containing nucleophiles (Scheme 1). The goal of this study was to develop a reliable chemical route to monocarboxyethyl functionalized fluorescein analogues of structure **1**.

Two approaches to the synthesis of asymmetric fluoresceins have been previously reported. One is the direct condensation of substituted phthalic anhydrides with a mixture of two

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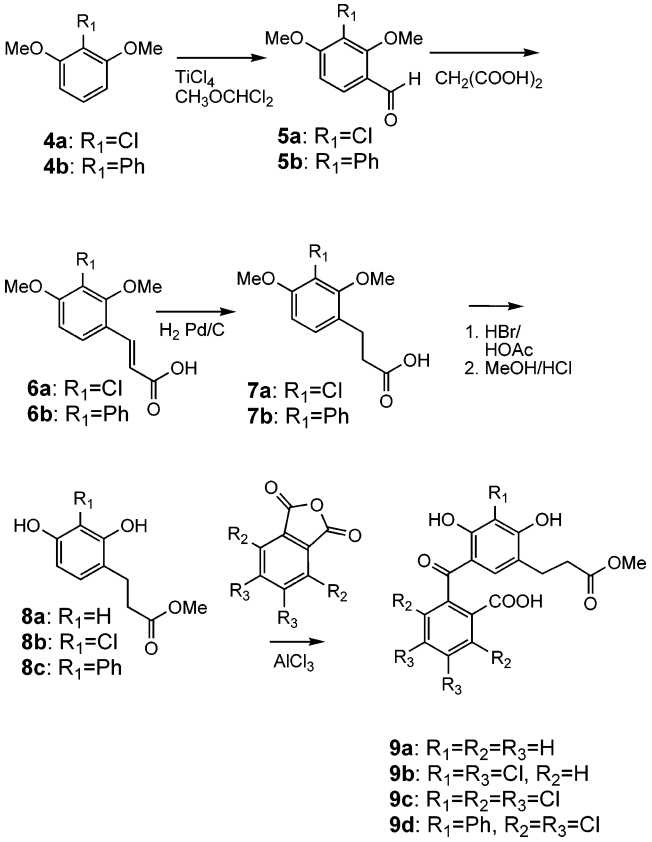
SCHEME 2. Synthesis of Substituted Benzophenone Dye Precursors



resorcinol analogues in the presence of a strong acid.[10] This method requires separation of several dyes and suffers from low product yields. The second approach is based on preparing the benzophenone precursor by reacting substituted phthalic anhydride with 1 equiv of a resorcinol analogue in the presence of aluminum chloride.[8,9] This is followed by a condensation with another resorcinol analogue to form the dye. The second method became the focus of our synthetic strategy because it offered a regiospecific way of dye assembly. The synthesis of the benzophenone intermediates is shown in Scheme 2. 2-Substitued 1,3-dimethoxybenzenes **4** were formylated using α,α-dichloromethylmethyl ether in the presence of $TiCl_4$ to afford benzaldehydes **5**. The Knoevenagel condensation with malonic acid produced cinnamic acids **6**. Catalytic reduction (H_2/Pd−C) of the double bond followed by demethylation of the phenolic groups (HBr/AcOH/H_2O or BBr_3/CH_2Cl_2 for **7b**) and esterification of the carboxy group furnished 2-substituted methyl 1,3-dihydroxyphenyl-4-propanoates **8** in 73−84% overall yields (starting from **4**). The Friedel−Crafts acylation with phthalic anhydride (or its substituted analogues) in the presence of $AlCl_3$ generated the desired benzophenone derivatives **9** in 60−80% yields.

A number of reaction conditions have been previously reported for condensation of 2,4-dihydroxy-2′-carboxybenzophenones with resorcinol analogues. They include the following: heating neat components[9] at 190 °C, sulfuric acid[11] at 140 °C, *p*-toluenesulfonic acid[12] at 103 °C, fusion with zinc chloride[9,13,14] at 150−250 °C or methanesulfonic acid[15] at 120−130 °C. All of those approaches require elevated (100−250 °C) temperatures. Among those listed above, methanesulfonic acid has become the most popular reagent[16] since it is a fairly good solvent and allows the reaction to be carried out at a moderately low temperature (80−100 °C). Therefore, our initial dye preparations were done in neat methanesulfonic acid. We later found that methanesulfonic acid can be used in a mixture with trifluoroacetic acid (TFA) to improve the solubility of the starting materials and facilitate the conversion of the methyl ester to a free carboxy group. With these initial conditions in our hands, condensation reactions between benzophenones **9** and resorcinols **10** were carried out (Scheme 3, route A). Unexpectedly, we found that, along with one main product, one or two additional side products were observed in many reactions. In the case of the simplest analogue **1a**, the side products were isolated and shown to be unsubstituted fluorescein (**11a**) and bis-2′,7′-(carboxyethyl)fluorescein[17] (**12a**). The formation of the symmetric side products can be explained by the retro-Friedel−Crafts fragmentation previously described for similar compounds.[14,18]

The formation of asymmetric fluoresceins is likely to proceed via an intermediate generation of a benzonium ion **B** (Scheme 4[19]), which should exist in equilibrium with carbonium ion **C**, followed by a cyclization (dehydration) step to furnish the hydroxyxanthenone ring. Under the reaction conditions (TFA/CH_3SO_3H, 80 °C) the intermediate **B** is able to reversibly generate two types of biphenylcarbonyl fragments **A** eventually leading to the formation of the symmetric side products **11** and **12**. The nature of substituents in both resorcinol parts of the triphenyl carbocation intermediate should influence which benzophenone fragments are formed during retro-acylation. Indeed, the same benzophenone **9b** affords a much higher yield of asymmetric dye when reacted with unsubstituted resorcinol **10a** compared to chloro-substituted **10b** and **10c** (reactions 4, 7, and 10 in Table 1). This can be explained by preferential formation of benzonium ion **B** with the positive charge located in the most electron-rich resorcinol ring, which in the case of the **9b** + **10a** combination is the non-chlorinated one. This will consequently lead to retro-formation of the starting benzophenone **9b**. The selectivity is lost when both resorcinol rings are nearly equivalent as seen in reactions 7 (**9b** + **10b)** and 10 (**9b** + **10c)** (Table 1).

Effects of substituents in the carboxyphenyl ring are less clear. Our results show that reaction of a pentachloro-substituted benzophenone **9c** with resorcinol **10b** (reaction 13) affords 97% of desired asymmetric dye **1e**. In contrast, its trichloro-substituted analogue **9b**, which lacks the R_2 chloro substituents in the carboxyphenyl ring, generates only about 44% of the

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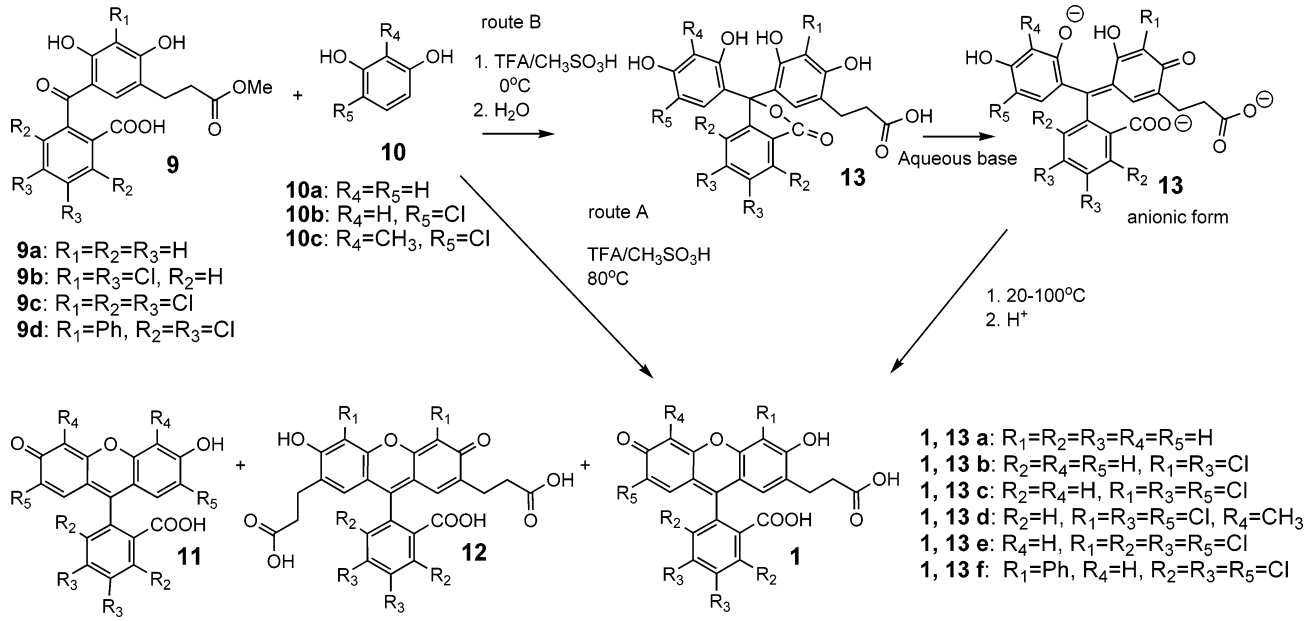
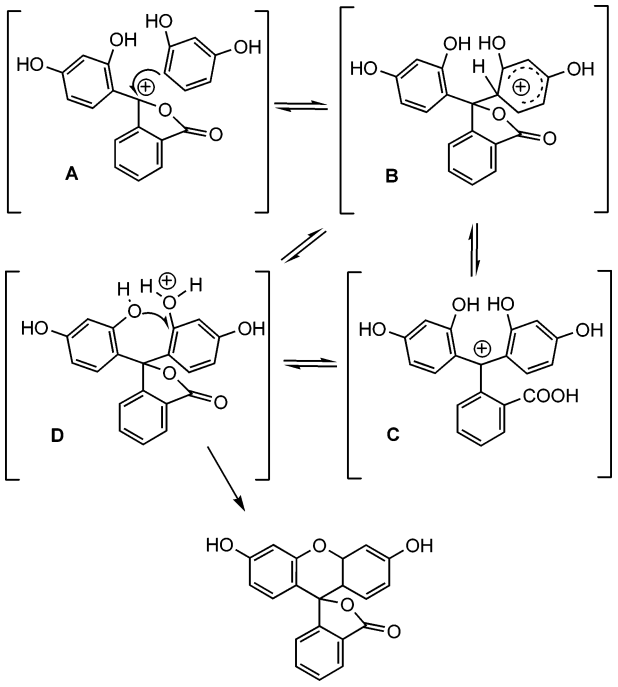
(19) No substituents are shown for simplicity. Not all possible intermediates are shown for simplicity. The lactone structure of intermediates A, B, D, and final dye may alternatively exist as a methanesulfonate adduct as proposed in ref 2b.

SCHEME 3. Two Synthetic Routes to Asymmetric 2′-Carboxyethyl-Substituted Fluoresceins



SCHEME 4. Proposed Mechanism for Dye Formation by Route A



target compound **1c** and more than 50% of symmetric byproduct **11c** (reaction 7). It is unlikely that such a dramatic change in reaction specificity is solely due to the electronic effects of the R_2 chloro substituents. One possible explanation is that steric effects attributable to the presence of the C-7 chloro atom in the vicinity of the crowded central carbon destabilize the nonplanar intermediate **B**, which is required for the fragmentation reaction. This conclusion is supported by published data for an asymmetric fluorescein containing a single chloro group in the C-7 position,[20] which was synthesized with no evident formation of symmetric side products.

In order to reduce the formation of symmetric byproducts we attempted to adjust the reaction conditions. A 2-fold decrease in the excess of the resorcinol component had only a limited effect on the amount of the side products (Table 1). We also attempted to carry out the reactions at lower temperatures (<20 °C). The formation of the carbocation **C** (Scheme 4) (observed as a dark-purple intermediate) was moderately fast and achievable at the lower temperatures. However, elevated temperatures were required to promote the cyclization. Unfortunately, these conditions also facilitated the undesired retro-Friedel−Crafts fragmentation and accumulation of the side products.

We discovered that this problem can be overcome if the dye preparation is carried out in two separate steps (Scheme 3, route B). The first step is done at 0 °C (TFA, CH_3SO_3H) for 10−15 h; the resulting lactones **13** are isolated by precipitation in cold water as off-white or lightly colored solids (due to spontaneous cyclization). The decrease in reaction temperature resulted in significant reduction of the acid-catalyzed fragmentation. The second step is carried out in a warm (20−100 °C) aqueous solution at a pH of 7−10 followed by acidification to precipitate the desired 2′-carboxyethyl-fluoresceins **1**. Since the cyclization step proceeds through an anionic form of **13**, it is not subject to retro-fragmentation even at elevated temperatures. The new conditions increased the fraction of desired asymmetric dyes up to 94−99% in all tested reactions.

In summary, we have developed a mild two-step synthesis of asymmetric 2′-carboxyethyl-substituted fluoresceins including ones that are not easily prepared using traditional approaches. The developed procedure should also be applicable to the preparation of a variety of other asymmetric fluorescein analogues. The method is especially useful when harsh acidic treatments are unacceptable due to either the instability of the intermediates or undesired, acid-catalyzed chemical rearrangements.

Experimental Section

Representative Procedure for Dye Preparation (Route A). 2-[2-(2-Carboxyethyl)-4,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl]-3,4,5,6-tetrachlorobenzoic Acid (1e). A suspension of **9c**

(20) Lukhtanov, E.; Vorobiev, A. U.S. Patent application US 2006/0199955 A1 2006.

TABLE 1. Effect of Reaction Conditions on Yields of 2′-Carboxyethylfluoresceins

reaction no.	resorcinol	benzophenone	target product	route	**10** to **9** ratio	distribution of products[a] (%)		
						1	**11**[b]	**12**[b]
1	**10a**	**9a**	**1a**	A	2	70.5	28.5	<1
2	**10a**	**9a**	**1a**	A	1	75.1	16.3	8.6
3	**10a**	**9a**	**1a**	B	1.5	94.1	5.8	<0.1
4	**10a**	**9b**	**1b**	A	2	83.2	16.8	<0.5
5	**10a**	**9b**	**1b**	A	1	84.4	15	<0.5
6	**10a**	**9b**	**1b**	B	1.5	99.6	<0.5	<0.1
7	**10b**	**9b**	**1c**	A	2	43.7	56.2	<0.5
8	**10b**	**9b**	**1c**	A	1	51.7	28.9	19.4
9	**10b**	**9b**	**1c**	B	1.5	95.7	4.3	<0.1
10	**10c**	**9b**	**1d**	A	2	18.9	80.6	0.5
11	**10c**	**9b**	**1d**	A	1	33.7	47.5	18.8
12	**10c**	**9b**	**1d**	B	1.5	94.5	4.5	<0.5
13	**10b**	**9c**	**1e**	A	2	97.1	2.5	<0.5
14	**10b**	**9d**	**1f**	A	2	98.2	1.5	<0.5

[a] Percent yields were calculated by analysis (260 nm) of reversed-phase HPLC profile of the reaction mixtures. [b] Except for **11a** and **12a**, structure assignments were based on reversed-phase C18 HPLC mobilities with **12** being the most hydrophilic (shorter elution time) and **11** the most hydrophobic (longer elution time) compared to corresponding asymmetric dye **1**.

(0.259 g, 0.5 mmol) and 4-chlororesorcinol (**10b**) (0.145 g, 1 mmol) in a mixture of TFA (1.25 mL) and methanesulfonic acid (1.25 mL) was heated with stirring at 80 °C for 2 h. The dark tan solution was cooled and diluted with water to precipitate the product. The solid was collected by filtration, washed with water, and dried. The crude material was chromatographed on silica in a gradient (5−20%) of MeOH in CH_2Cl_2 (+10% triethylamine). The pure product fractions were concentrated. The resulting solid was suspended in water, acidified with 1 N HCl to a pH of ∼2, filtered, and washed with water. Drying under vacuum (over P_2O_5) afforded 0.25 g (82%) of the title dye **1e** as an orange solid. ^{1}H NMR (300 MHz, CD_3OD + 0.8% NaOD +1.2% D_2O): δ 7.08 (s, 1H), 6.85 (s, 1H), 6.68 (s, 1H), 2.56 (m, 2H), 2.48 (m, 2H). ^{13}C NMR (75.5 MHz, CD_3OD + 0.8% NaOD +1.2% D_2O): δ 182.3, 177.5, 175.2, 169.7, 157.6, 154.3, 148.0, 135.2, 132.6, 132.4, 130.2, 129.6, 128.8, 128.7, 127.2, 113.3, 110.7, 110.0, 105.1, 38.5, 29.4. HRMS (FTMS) (*m*/*z*) calcd for $C_{23}H_9Cl_6Na_2O_7$ $(M - H + 2Na)^+$ 652.8269, found 652.8293.

Representative Procedure for the Preparation of Dyes (Route B). 2-[2-(2-Carboxyethyl)-6-hydroxy-3-oxo-3*H*-xanthen-9-yl]-benzoic Acid (1a). A solution of **9a** (4.8 g, 14 mmol) and resorcinol (**10a**) (2.3 g, 21 mmol) in 70 mL of TFA was prepared and cooled to 0 °C using an ice/water bath. To this solution were slowly added 25 mL of methanesulfonic acid maintaining the temperature at 0−2 °C. The reaction was stirred at 0 °C for 15 h and poured onto 500 g of ice. Precipitated material was collected by filtration, resuspended in water (500 mL), and treated with triethylamine to a pH of ∼10. The resultant dark-brown solution was heated to boiling and slowly cooled to room temperature. It was then acidified with concentrated hydrochloric acid to a pH of ∼2 and extracted with ethyl acetate (5 × 100 mL). The extract was washed with brine and dried over Na_2SO_4. Concentration gave a tan, viscous oil, which solidified upon treatment with water (∼100 mL). Filtration and drying over P_2O_5 *in vacuo* afforded 5.1 g (90%) of **1a** as an orange solid. An analytical sample was purified by silica gel chromatography as described for **1e**. ^{1}H NMR (300 MHz, CD_3OD + 0.8% NaOD +1.2% D_2O): δ 8.03 (m, 1H), 7.75 (m 2H), 7.57 (m, lH), 6.99 (m, 2H), 6.6−6.4 (m, 3H), 2.74 (m, 2H), 2.33 (m, 2H). ^{13}C NMR (75.5 MHz, CD_3OD + 0.8% NaOD +1.2% D_2O): δ 182.7, 182.5, 180.7, 174.3, 160.0, 159.8, 159.3, 141.3, 137.2, 135.0, 131.8, 130.9, 130.2, 129.8, 129.7, 122.9, 113.5, 112.9, 104.3, 104.0, 38.6, 28.8. HRMS (FTMS) (*m*/*z*) calcd for $C_{21}H_{17}O_7$ $(M + H)^+$ 405.0945, found 405.0956.

Acknowledgment. We thank N. Scarr for useful discussions and help with compound characterization.

Supporting Information Available: Experimental details and characterization data for compounds **1b**, **1c**, **1d**, **1f**, **5b**, **6a**, **6b**, **7a**, **7b**, **8a**−**c**, **9a**−**d**, and **10c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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