Synthesis of Fluorinated Fluoresceins

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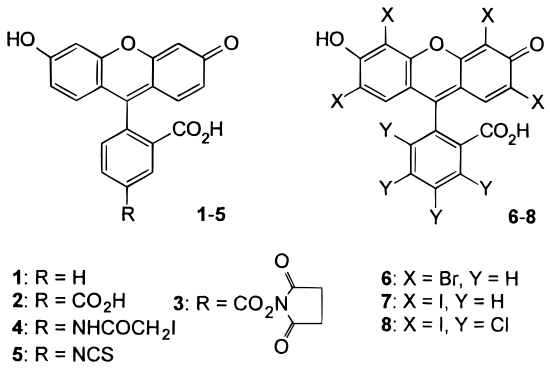
Several novel fluorinated fluoresceins (Oregon Green dyes) were prepared by the reaction of fluororesorcinols with phthalic anhydride and its derivatives. A novel regiospecific synthesis of fluororesorcinols was key to the successful synthesis of these new fluorophores. (Polyfluoro)-nitrobenzenes were reacted with 2 equiv of sodium methoxide followed by reduction, hydrodediazoniation, and demethylation, giving the first straightforward synthesis of 2-fluororesorcinol, 4-fluororesorcinol, 2,4-difluororesorcinol, and 2,4,5-trifluororesorcinol. These fluorinated fluoresceins have higher photostability and ionize at a lower pH (pK_a = 3.3−6.1) than fluorescein (pK_a = 6.5). Some of the fluorinated fluoresceins have very high quantum yields (0.85−0.97), which, in combination with their lower pK_as and high photostability, makes them superior fluorescent dyes for use as reporter molecules in biological systems.

Introduction

The preparation of fluorescein (**1**) by the condensation of resorcinol with phthalic anhydride catalyzed with zinc chloride was first reported in 1871 by von Bayer.[1] Fluorescein is a highly fluorescent molecule that absorbs light at 492 nm and emits at 517 nm in water, with a quantum yield of 0.92 at pH > 8.[2] Over the years a wide variety of fluorescein derivatives have been prepared and used as fluorescent detection reagents. For example, 5(6)-carboxyfluorescein (**2**), 5(6)-carboxyfluorescein succinimidyl ester (**3**), 5-iodoacetamidofluorescein (**4**), and fluorescein isothiocyanate (**5**, FITC) are among the most widely used fluorescent derivatization reagents in cytology and immunohistochemistry (Chart 1).[3] Fluorescein derivatives are particularly suitable for, but not limited to, biological experiments in which fluorescein is covalently attached to materials such as peptides, proteins (especially antibodies), nucleotides, oligonucleotides, drugs, hormones, lipids, and other biomolecules.[4]

Despite their widespread use in biological assays, fluorescein-based dyes have well-known deficiencies. In particular, fluorescein conjugates are unstable with respect to the intense illumination produced in most fluorescence instrumentation.[5] The result is irreversible photobleaching, which typically results in a rapid decrease in fluorescent signal. A second problem of fluorescein is that in aqueous solution it can exist in cationic (**9**), neutral (**1**), anionic (**10**), and dianionic (**11**) forms (Scheme 1), making its absorption and fluorescence properties strongly pH dependent.[6] The pK_a of fluorescein (**1**) in water is approximately 6.4;[7] at the physiological pH range a considerable population of fluorescein is in the protonated, nonfluorescent form[8] (**12** and **13** in Scheme 1). A third difficulty that is recognized when using fluorescein dyes is the tendency of their protein conjugates to exhibit quenched fluorescence relative to that of the free fluorophore. This typically leads to the protein conjugates of fluorescein exhibiting less fluorescence even when increasing numbers of dye molecules are conjugated to the protein,[9] reducing the sensitivity that is possible for assays using fluorescein conjugates.

Chart 1. Fluorescein Derivatives



The substitution of hydrogen atoms by fluorine atoms in organic compounds often results in profound changes in their properties,[10] largely due to the highly electronegative nature and small van der Waals radius of the fluorine atom. Unlike other halogenated compounds, in which an expected pattern derived from the substitution effect could be extrapolated, fluorination of organic compounds often results in products with unexpected properties. In the family of halogenated fluoresceins (Chart 1), 2′,4′,5′,7′-tetrabromofluorescein (eosin, **6**), 2′,4′,5′,7′-tetraiodofluorescein (erythrosin, **7**), and 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein (rose bengal, **8**[11]) were prepared shortly after the original synthesis of fluorescein. These halogenated derivatives undergo significant intersystem crossing to the triplet state after

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(1) von Bayer, A. *Chem. Ber.* **1871**, *5*, 255.
(2) Guilbault, G. G. In *Practical Fluorescence*; Guilbault, G. G., Ed.; Marcel Dekker Inc.: New York, 1990; Chapter 1.
(3) For convenience, only 5-isomers are shown; the numbering system is depicted in Scheme 1.
(4) For a source book and comprehensive references, see: Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed.; Molecular Probes Inc.: Eugene, OR, 1996.
(5) (a) Song, L.; Hennink, E. J.; Young, T.; Tanke, H. J. *Biophys. J.* **1995**, *68*, 2588. (b) Song, L.; Varma, C. A. G. O.; Verhoeven, J. W.; Tanke, H. *Biophys. J.* **1996**, *70*, 2959.
(6) Sjoback, R.; Nygern, J.; Kubista, M. *Spectrochim. Acta Part A* **1995**, *51*, L7.
(7) Guilbault, G. G. In *Practical Fluorescence*; Guilbault, G. G., Ed.; Marcel Dekker Inc.: New York, 1990; Chapter 6.
(8) *Ibid.*; pp 129−141.
(9) (a) Chapple, M. R.; Johnson, G. D.; Davidson, R. S. *J. Microsc.* **1990**, *159*, 254. (b) Chen, R. F.; Knutson, J. R. *Anal. Biochem.* **1988**, *172*, 61.
(10) Lentz, D. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1315 and references cited therein.
(11) (a) Schultz. *Farbstofftabellen*; Akademisch Verlagsgesellschaft m.b.H.: Leipzig, 1885. (b) Review: Neckers, D. C. *J. Photochem. Photobiol. A: Chem.* **1989**, *47*, 1.

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Scheme 1. pH Dependence of Fluorescein Equilibria

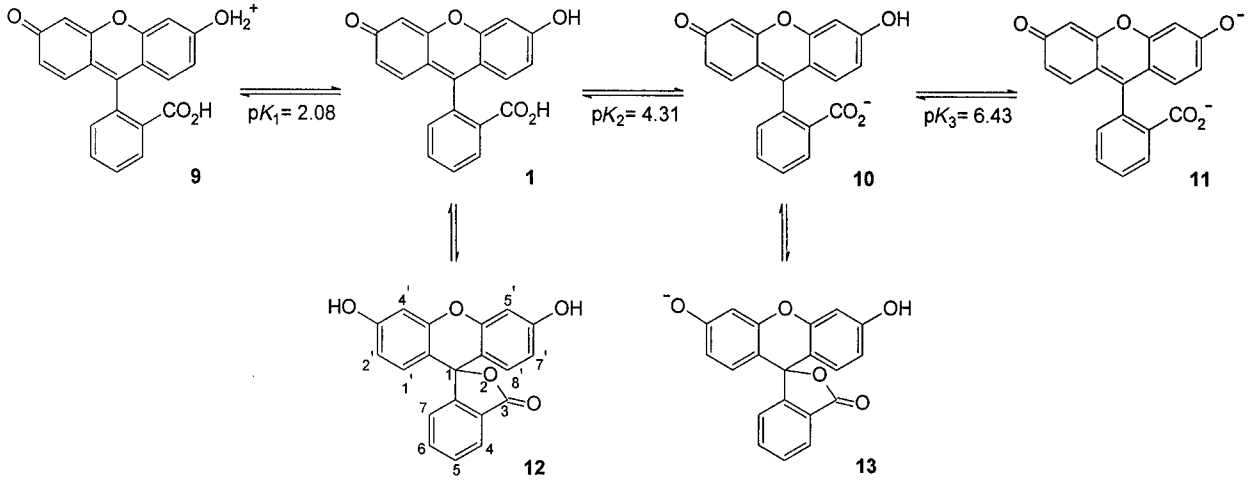
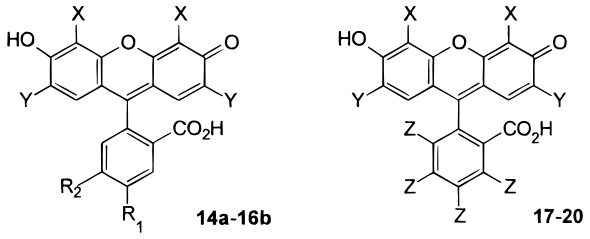


Chart 2. Fluorinated Fluoresceins



14: X = H, Y = F
15: X = F, Y = H
16: X = Y = F

a: R_1 = CO_2H, R_2 = H
b: R_1 = H, R_2 = CO_2H

17: X = Z = H, Y = F
18: X = Y = H, Z = F
19: X = H, Y = Z = F
20: X = Y = Z = F

light absorption. Hence, they have much lower fluorescence quantum yields than fluorescein but can serve as efficient sensitizers for singlet oxygen.[11b,12]

In our search for substitutes that improve upon the properties of fluorescein, we sought to retain the favorable characteristics of fluorescein, including high absorbance and good fluorescence, the ability to be excited using the 488-nm line of the argon-ion laser, and the ability to use standard optical filters for fluorescein that are already available for most fluorescence microscopes while simultaneously possessing characteristics that improve on the deficiencies of fluorescein. Fluorination of fluorescein permits us to examine the influence of fluorine on this widely used fluorescent molecule. To our knowledge, fluorinated fluorescein derivatives have not been reported in the literature; thus, we set out to determine if fluorination of fluorescein would help achieve our objectives. We now wish to report the synthesis and photophysical properties of 5(6)-carboxy-2′,7′-difluorofluorescein (**14a**,**b**), 5(6)-carboxy-4′,5′-difluorofluorescein (**15a**,**b**), 5(6)-carboxy-2′,4′,5′,7′-tetrafluoroluorescein (**16a**,**b**), 2′,7′-difluorofluorescein (**17**), 4,5,6,7-tetrafluorofluorescein (**18**), 2′,4,5,6,7,7′-hexafluorofluorescein (**19**), and 2′,4,4′,5,5′,6,7,7′-octafluorofluorescein (**20**) (Chart 2).

Results and Discussion

There were envisioned two potential approaches to the synthesis of fluorinated fluoresceins. One is the direct fluorination of fluorescein; the other is fluorination of the building blocks of fluorescein. There are several active positions on fluorescein that are readily available for direct fluorination by electrophilic fluorination reagents.[13] However, the isolation of pure products proved to be a serious problem. Thus, we examined the regiospecific synthesis of fluorinated resorcinols as a means to achieve the regiospecific synthesis of various fluorinated fluoresceins. Durrani and Tyman reported the synthesis of 2,4-dimethoxyfluorobenzene, which is a precursor of 4-fluororesorcinol, from 2,4-dimethoxyaniline by the classic Baltz−Schiemann reaction in 59% yield.[14] In our hands the best yield of the reaction was only 22%, and the purification was complicated by the formation of 1,3-dimethoxybenzene. The low yield was probably due to the presence of the *o*-methoxy group, as was reasoned by Suschitzky based on the observation of several *o*-substituted compounds.[15] Direct fluorination of resorcinol with cesium fluoroxysulfate reportedly gave a mixture of 2- and 4-fluororesorcinol in moderate yields.[16] We have carried out the fluorination of 1,3-dimethoxybenzene with Selectfluor (1-chloromethyl-4-fluoro-1,4-diazabicyclo[2.2.2]-octane bis(tetrafluoroborate)).[13f−h] The products were a mixture of 2,4- and 2,6-dimethoxyfluorobenzene.[17]

Because of these difficulties, we developed a general synthesis of fluorinated resorcinols that begins with fluorinated nitrobenzenes and results in excellent yields of the pure products. The purpose of the nitro group was to direct fluoride displacement by methoxy groups at positions *ortho* and *para* to it, thus putting the resorcinol framework into place. The pattern of fluoro substitution

(12) Linden, S. M.; Neckers, D. C. *J. Am. Chem. Soc.* **1988**, *110*, 1257.

(13) For fluorination with *N*-fluoropyridinium salts, see: (a) Umemoto, T.; Fukami, S.; Tomizawa, G.; Harasawa, K.; Kawada, K.; Tomita, K. *J. Am. Chem. Soc.* **1990**, *112*, 8563. (b) Umemoto, T.; Tomizawa, G. *J. Org. Chem.* **1995**, *60*, 6563. With *N*-fluoro-*N*-(phenylsulfonyl)benzenesulfonamide and related compounds, see: (c) Differding, E.; Ofner, H. *Synlett* **1991**, 187. (d) Singh, S.; DesMarteau, D. D.; Zuberi, S. S.; Witx, M.; Huang, H.-N. *Synlett* **1987**, *109*, 7194. (e) Barnette, W. E. *J. Am. Chem. Soc.* **1984**, *106*, 452. With Selectfluor, see: (f) Banks, R. E. U.S. Patent 5086178, 1992. (g) Banks, R. E.; Mohialdin-Khaffaf, S. N.; Lal, G. S.; Sharif, I.; Syvret, G. *J. Chem. Soc., Chem. Commun.* **1992**, 596. (h) Lal, G. S. *J. Org. Chem.* **1993**, *58*, 2791.

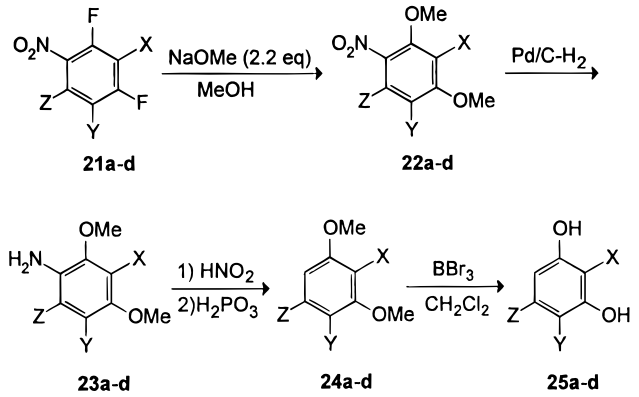
(14) Durrani, A. A.; Tyman, J. H. P. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1658.

(15) Suschitzky, H. *Adv. Fluorine Chem.* **1965**, *1*, 10. An improved method for fluorodediazoniation of arylamines substituted with a polar group (including an *o*-methoxy group) was recently reported; see: Sasaki, K.; Oishi, M.; Imaki, N. *J. Fluorine Chem.* **1996**, *76*, 59.

(16) Patrick, T. B.; Darling, D. L. *J. Org. Chem.* **1986**, *51*, 3242.

(17) During the preparation of this manuscript J. J. Yang, R. L. Kirchmeier, and J. M. Shreeve reported similar results, which appeared at: Thirteenth Winter Fluorine Conference, St. Petersburg Beach, FL, Jan. 19−24, 1997; Poster P32.

Scheme 2. Synthesis of Fluorinated Resorcinols

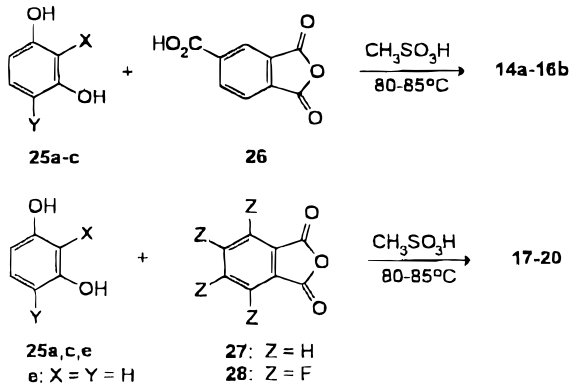


in the desired resorcinol was determined by which fluoro substituents were left after bis-methoxide addition. The directing nitro group was then removed by catalytic hydrogenation followed by hydrodediazoniation. Demethylation generated the desired fluorinated resorcinols. For example, synthesis of 4-fluororesorcinol (**25a**) was accomplished in four steps starting from 5-nitro-1,2,4-trifluorobenzene (**21a**) with an 80% overall yield (Scheme 2). Thus, reaction of **21a** with 2.2 equiv of NaOMe in MeOH gave 2,4-dimethoxy-5-fluoronitrobenzene (**22a**) in quantitative yield. Reduction of the nitro group was also achieved in quantitative yield with Pd/C−H_2 in EtOAc. Hydrodediazoniation of **23a** with HNO_2/H_3PO_2 yielded **24a** in 87% yield. Treatment of **24a** with BBr_3 in CH_2Cl_2 for 24 h gave 4-fluororesorcinol (**25a**) in 92% yield. The same strategy was applied to the synthesis of 2-fluororesorcinol (**25b**), 2,4-difluororesorcinol (**25c**), and 2,4,5-trifluororesorcinol (**25d**), all beginning with the appropriate commercially available fluorinated nitrobenzenes (**21b**−**d**) (Scheme 2).

Traditional fluorescein synthesis has been carried out by fusion of resorcinol, phthalic anhydride, and zinc chloride at high temperature. This reaction was not practical for microscale synthesis, since considerable amounts of the starting materials were lost due to sublimation. It was found that methanesulfonic acid (CH_3SO_3H) served as both a suitable solvent and a Lewis acid catalyst in the dye-forming reaction, giving an improved procedure with higher yields of products under milder conditions.[18] For example, heating difluororesorcinol **25c** and tetrafluorophthalic anhydride (**28**) in CH_3SO_3H at 85 °C for 48 h gave octafluorofluorescein **20** in 65% isolated yield (Scheme 3). The same reaction carried out in molten $ZnCl_2$ at 170−180 °C for 20 min gave only 17% yield of **20**. Similarly, using CH_3SO_3H as the solvent, **15**−**20** were synthesized in 60−92% yields. Interestingly, 5-fluororesorcinol[19] and 2,4,5-trifluororesorcinol (**25d**) failed to give fluorescein derivatives upon reaction with phthalic anhydride. Often the fluoresceins thus obtained were converted to the diacetate form for easy recrystallization or column chromatography purification and then hydrolyzed back to the free dyes (Scheme 4).

The wavelength of absorption maximum (Abs), fluorescence wavelength maximum (Em), fluorescence quantum yield (Φ_f), photostability, pK_a, and extinction coefficient (ϵ) are summarized in Table 1 for all the novel fluorinated fluoresceins. For comparison, the photophysical properties of fluorescein (**1**) and carboxyfluorescein (**2**) are also included. In general, fluorination resulted in a bathochromic shift of both Abs and Em, but the shift was much smaller than for other halogen substitutions.[20] Unanticipated was that the substitution at the 2′ and 7′ positions had no effect on the Abs/Em. The following pairs, with the latter entry having two additional fluorines at the 2′ and 7′ positions, have the same Abs/Em maximum: **1** vs **17**, **2** vs **14**, **15** vs **16**, and **18** vs **19**. In fact, the absorption spectra of 5-carboxyfluorescein (**2**) and 5-carboxy-2′,7′-difluorofluorescein (**14a**) matched perfectly beyond 360 nm after normalization to the same peak intensity (Figure 1).

Scheme 3. Synthesis of Fluorinated Fluoresceins



Scheme 4. Purification of Fluorinated Fluoresceins via the Diacetate

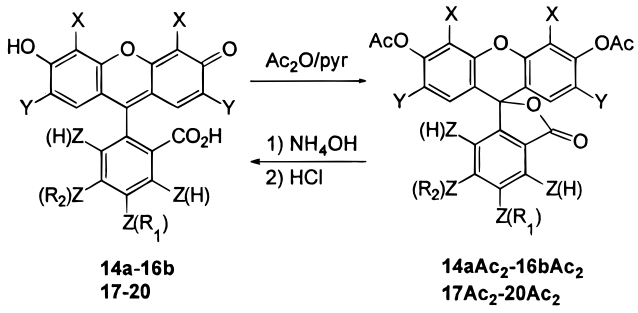


Table 1. Physicochemical Properties of Fluorinated Fluoresceins[a]

compd	Abs/Em (nm)	QY[b]	bleaching[c]	pK_a[d]	ϵ (cm^{-1} M^{-1})[e]
1	490/514	0.92	17	6.5	90 000
2	492/516	0.92	17	6.4	84 700
17	490/514	0.97	8	4.8	82 400
14a	492/517	0.92			
14b	492/514	0.92			
14a,b	492/516	0.92	9	4.8	85 900
15a,b	510/534	0.43	11	5.2	84 200
16a,b	510/534	0.59	6	3.7	78 100
18	508/527	0.85	7	6.1	85 600
19	508/527	0.96	4	4.5	81 200
20	535/553	0.47	8	3.3	83 400

[a] Determined in pH 9.0 phosphate buffer, except for pK_a. [b] Quantum yield. [c] Percentage of fluorescence lost after 33 min of irradiation in a fluorometer at the wavelength of maximum excitation.[24] [d] Data obtained from a double-reciprocal plot of pH vs fluorescence emission intensity titration curve. [e] Extinction coefficient.

(18) Confalone, P. N. *J. Heterocycl. Chem.* **1990**, *27*, 31.

(19) Obtained in one step from commercially available 3,5-dimethoxyfluorobenzene.

(20) For example: Abs/Em for 2′,7′-dichlorocarboxyfluorescein = 540/529 nm; 2′,4′,5′,7′-tetrabromofluorescein = 520/543 nm; 2′,4′,5′,7′-tetraiodofluorescein = 528/553 nm.

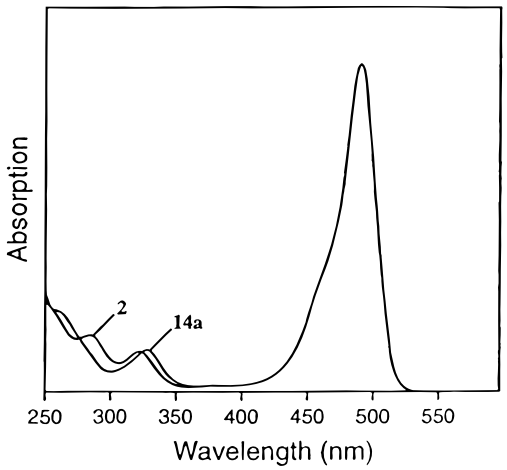


Figure 1. Normalized absorption spectra of **2** and **14a** (pH 9.0).

The protolytic equilibrium constants of fluorescein of the cation, neutral, anion, and dianion forms (Scheme 1) are reported to be $pK_1 = 2.08$, $pK_2 = 4.31$, and $pK_3 = 6.43$.[5] For our interests we will limit the discussion to pK_3, which is the pK_a of the phenol.[21] Because the dianion (**11**) is the most intense fluorescence species, factors that affect the pK_a of the phenol will have the largest impact on the fluorescence properties of fluorescein. It is well documented that fluorinated phenols have lower pK_a values than their parent compounds because of the strong electron-withdrawing ability of the fluorine.[22] Similarly, the fluorinated fluoresceins were found to have pK_a values 0.4−3.2 pK_a units lower than fluorescein. Fluorination at the 4, 5, 6, and 7 positions lowers the pK_a by about 0.4 unit (**1** vs **18**). Fluorination at the 4′ and 5′ positions lowers the pK_a by about 1.2 units (**2** vs **15**). Fluorination at the 2′ and 7′ positions lowers the pK_a by about 1.6 units (**2** vs **14**). Interestingly, the decrease in the pK_a by further fluorination at specific positions appears to have an additive effect. For example, 2′,4′,5′,7′-tetrafluorofluorescein (**16**) has a pK_a of 3.7, which approximates the combined effect of fluorination at the 2′ and 7′ positions in compound **14** (1.6 units lower than **2**) and at the 4′ and 5′ positions in compound **15** (1.2 units lower than **2**). The estimated pK_a for **16** would be 6.4 − 1.6 − 1.2 = 3.6. Similarly, 2′,4,5,6,7,7′-hexafluorofluorescein (**19**) has a pK_a of 4.5, which is about equal to the combined effect of 2′,7′-difluorination and 4,5,6,7-tetrafluorination. Using **1** as the parent compound, the estimated pK_a for **19** was 6.5 − 1.6 − 0.4 = 4.5. Also, the pK_a for 2′,4,4′,5,5′,6,7,7′-octafluorofluorescein (**20**) is 3.3, which is approximately the combined effect of 3-fold fluorination (6.5 − 1.2 − 1.6 − 0.4 = 3.3).

Photobleaching is a dynamic process by which a fluorophore undergoes a photoinduced chemical destruction upon exposure to light and thus loses its ability to fluoresce. The photobleaching mechanism for a fluorophore present in biological systems is a complicated process that has been the focus of many studies.[23] For fluorophores in solution, the chemical environment is well controlled, and single-exponential bleaching is generally observed. We have measured the fluorescence intensity of these fluorinated fluoresceins in a spectrofluorometer at their excitation maximum[24] for 33 min (Table 1). A single-exponential bleaching curve was observed for each of the fluorophores measured. All of the fluorinated fluoresceins have significantly slower photobleaching rates than fluorescein. It has been shown that the introduction of fluorine-containing substituents makes it possible to obtain dyes that are more resistant to oxidation, light, heat, and other physical effects.[25] Our results are consistent with the reported properties of other fluorine-containing molecules. In general, the photostability of the fluorinated fluoresceins improves as the number of fluorine atoms increases.

There are three primary processes involved after a dye molecule is excited: *i.e.*, fluorescence, intersystem crossing, and internal conversion; the rate constants are k_f, k_{isc}, and k_{ic}, respectively. They are related as follows:

$$\tau_f = 1/(k_f + k_{isc} + k_{ic})$$

$$\Phi_f = k_f/(k_f + k_{isc} + k_{ic})$$

$$\Phi_t = k_{isc}/(k_f + k_{isc} + k_{ic})$$

where τ_f is the fluorescence lifetime, Φ_f is the fluorescence quantum yield, and Φ_t is the triplet quantum yield.

For fluorescein: $k_f = 2.134 \times 10^8\ s^{-1}$, $k_{isc} = 6.6 \times 10^6\ s^{-1}$, and $k_{ic} = 50\ s^{-1}$.[26] Lindqvist reported that the long-lived triplet, not the short-lived singlet, excited state was responsible for most of the photochemical activity of fluorescein.[27] This was further demonstrated by Gollnick *et al.* who showed the fate of the singlet excited state of xanthene dyes had no measurable difference even in oxygen-saturated solutions.[28] There are many major reactions involved the photoinduced triplet state, which come exclusively from the singlet excited state through intersystem crossing (k_{isc}). Three of the reactions that could lead to irreversible bleaching photoproducts are (1) the reactions of two triplet dyes to form semireduced (R) and semioxidized (X) form of dyes; (2) the reaction of triplet dye with ground-state dye to form R and X; (3) the reaction of triplet-state dye with oxygen to form X and HO_2 (or O_2^-). The first two reactions represent the occurrence of an electron-transfer process; the third reaction is chemical quenching by oxygen. A slower rate of bleaching indicates that at least one of the three reactions is affected assuming other conditions remain the same.

The fluorescence lifetimes of both **14a** and **19** were found to be 4.1 ns, which is identical to that of fluorescein reported by Sjoback *et al.*[6] In addition, the absorption, emission, and quantum yield of **14** were identical to that of fluorescein. These data strongly indicate that the

(21) For some compounds in this paper there are four ionizable protons within a molecule; however, we will focus on the pK_a of the 6′-phenol.

(22) (a) Fujio, M.; McIver, R. T., Jr.; Taft, R. W. *J. Am. Chem. Soc.* **1981**, *103*, 4017. (b) Dolbier, W. R., Jr.; Burkholder, C.; Abboud, K. A.; Loehle, D. *J. Org. Chem.* **1994**, *59*, 7688.

(23) Reference 5a and references cited therein.

(24) The band pass was 20 nm and centered at the excitation maximum.

(25) (a) Yagupol'skii, L. M.; Il'chenko, A. Ya.; Gandel'sman, L. Z. *Russ. Chem. Rev.* **1993**, *52*, 993. (b) Herd, K. J. Fluorine-containing dyes In *Organofluorine Chemistry: Principles and Commercial Applications*; Banks, R. E., *et al.*, Eds.; Plenum Press: New York; Chapter 13.

(26) Tsien, R. Y.; Waggoner, A. Fluorophores for confocal microscopy: photophysics and photochemistry. In *The Handbook for Biological Confocal Microscopy*; Pawley, J., Ed.; IMR Press: Madison, WI, 1994; pp 153−161.

(27) Lindqvist, L. *Arkiv Kemi.* **1960**, *16*, 79.

(28) Gollnick, K.; Franken, T.; Fouda, M. F. R.; Paur, H. R.; Held. S. *J. Photochem. Photobiol. B: Biol.* **1992**, *12*, 57.

introduction of fluorines at the 2′ and 7′ positions of fluorescein (*i.e.*, **14a**) does not alter the decay pathways of the excited singlet species. The difference in bleaching rates most likely is associated with the fate of the triplet species. There are two possible explanations that account for the improved resistance to photobleaching: (1) The introduction of fluorines at the 2′ and 7′ positions could shorten the triplet lifetime, thus decreasing the probability of its reaction with a quencher. (2) The differences in triplet lifetimes are not significant, but the incorporation of fluorine atoms into fluorescein inhibits the chemical reactions involving the triplet state, including the three irreversible bleaching reactions described above. These two possibilities could be present simultaneously without contradicting each other.

The fluorescence quantum yields of these fluorinated fluoresceins are similar to that of fluorescein (**1**). A general observation is that fluorination at the 2′ and 7′ positions has little or no effect on the quantum efficiency, but substitution at the 4′ and 5′ positions decreases the quantum yield. Fluorination of the carboxyphenyl ring has somewhat less effect on the quantum efficiency. The succinimidyl ester of compound **14a** and the carboxymethyl thioether of **19**[29] were prepared for evaluation of their protein conjugation. Among other biomolecules, goat anti-mouse and anti-rabbit IgGs, avidin, streptavidin, protein A, concanavalin A, wheat germ agglutinin, and phalloidin have been labeled with these two dyes. The resulting conjugates are significantly more fluorescent at the same fluorophore/protein ratio (F/P) than fluorescein-labeled conjugates.[30] This finding indicates that use of these fluorinated fluoresceins can yield more fluorescent conjugates because of their greater resistance to quenching. In general, quenching can be defined as a bimolecular process that reduces the fluorescence quantum yield without major change in the fluorescence emission spectrum. In the case of protein conjugates, two types of quenching are recognized as follows: (1) quenching by protein-to-dye interaction, *i.e.*, fluorescence per dye is smaller than that of the free dye in solution, and (2) quenching by dye-to-dye interaction, *i.e.*, loss of incremental fluorescence enhancement per bound dye. For conjugates of the carboxymethyl thioether of **19** no significant quenching of type 2 is observed even after a F/P of 13 was reached. In the case of FITC (**5**) conjugates, quenching is significant when a F/P of 5 was reached. The nature of this diminished quenching is of interest to us and is currently under investigation.

Conclusion

We have developed an efficient method for the regiospecific synthesis of fluorinated fluoresceins. The 5(6)-carboxy-2′,7′-difluorofluoresceins (**14a**,**b**) are the best replacements for the widely used carboxyfluorescein (**2**) and fluorescein isothiocyanate (**5**), since the absorption and emission are virtually unchanged. Also, they can be excited with the 488-nm spectral line of the argon-ion laser used in flow cytometers and confocal laser scanning microscopes. The lower pK_a, increased resistance to photobleaching, high quantum yield, and diminished quenching on protein conjugation make the dyes superior to other fluoresceins for bioconjugation. Hexafluorofluorescein **19** has a longer wavelength emission spectrum than fluorescein. Furthermore, its pK_a, photostability, and high quantum yield also make it a fluorescent dye particularly suitable for the 514-nm line of the argon-ion laser.

Experimental Section

General. The ^{1}H-NMR and ^{19}F-NMR spectra were recorded in the same solvent as indicated at 400 and 282 MHz, respectively. Chemical shifts for ^{1}H-NMR are reported in ppm downfield from TMS (δ). Chemical shift for ^{19}F-NMR are reported in ppm upfield from $CFCl_3$ (ϕ). Coupling constants (J) are reported in hertz. Melting points are corrected relative to known standards.

Materials. Sodium methoxide (25% wt solution in MeOH), poly(fluoronitrobenzene)s (**21a**−**d**), 4 M HCl in dioxane, H_3PO_2 (50% aqueous solution), BBr_3 (1 M in CH_2Cl_2), anhydrous MeOH, THF, and CH_2Cl_2 were obtained from Aldrich Chemical Co. CH_3SO_3H was obtained from Fluka Chemical Co.

Method A. General Procedure for the Synthesis of Dimethoxyfluoronitrobenzenes. Sodium methoxide (2.2 equiv, 25% in MeOH) was added dropwise to a solution of **21** (1.0 equiv) in MeOH (0.3−0.4 M) under nitrogen at 4 °C. The resulting reaction mixture was stirred at room temperature for 1−24 h, with progress monitored by TLC. The reaction was then quenched with 1 M citric acid (0.1 equiv) and the MeOH was removed *in vacuo*. The residue was taken up in ether, washed with 1 M citric acid (2×) and brine, dried (Na_2SO_4), concd *in vacuo*, and purified by flash column chromatography (EtOAc/hexane) or recrystallization to yield the dimethoxyfluoronitrobenzenes.

Method B. Same as method A, except the reaction mixture was heated under reflux for 2−6 h.

2,4-Dimethoxy-5-fluoronitrobenzene (22a). Following method B, compound **21a** (70.7 g, 0.40 mol) gave, after recrystallization from EtOH (1500 mL), **22a** (76.92 g, 96%) as a colorless powder: mp 146−149 °C; ^{1}H-NMR (DMSO-d_6) δ 7.96 (dd, J = 11.4, 1H), 7.01 (d, J = 7.3, 1H), 4.00 (s, 3H), 3.98 (s, 3H); ^{19}F-NMR ϕ 139.22 (dd, J = 7.0, 13.5). Anal. Calcd for $C_8H_8FNO_4$: C, 47.77; H, 4.01; N, 6.96. Found: C, 47.81; H, 3.96; N, 6.84.

2,4-Dimethoxy-3-fluoronitrobenzene (22b). Following method A, compound **21b** (5.53 g, 31.2 mmol) gave **22b** (6.30 g, 99%) as a pale yellow crystalline solid. An analytical sample was obtained by crystallization from light petroleum ether/CH_2Cl_2: mp 59−61 °C; ^{1}H-NMR ($CDCl_3$) δ 7.72 (dd, J = 9.5, 2.2, 1H), 6.71 (dd, J = 9.4, 7.5, 1H), 4.08 (s, 3H), 3.95 (s, 3H); ^{19}F-NMR ϕ 149.1 (d). Anal. Calcd for $C_8H_8FNO_4$: C, 47.77; H, 4.01; N, 6.96. Found: C, 47.64; H, 4.05; N, 6.80.

3,5-Difluoro-2,4-dimethoxynitrobenzene (22c). Following method A, compound **21c** (9.8 g, 50.0 mmol) gave **22c** (10.92 g, 99%) as a pale yellow solid: mp 32.0−32.5 °C; ^{1}H-NMR ($CDCl_3$) δ 7.52 (dd, J = 11.0, 2.2, 1H), 4.12 (s, 3H), 4.04 (s, 3H); ^{19}F-NMR ϕ 132.0 (m, 1F), 141.9 (d, 1F). Anal. Calcd for $C_8H_7F_2NO_4$: C, 43.85; H, 3.22; N, 6.39. Found: C, 43.84; H, 3.15; N, 6.15.

2,4-Dimethoxy-3,5,6-trifluoronitrobenzene (22d). Following method B, compound **21d** (5.00 g, 23.5 mmol) gave **22d** (5.56 g, 98%) as a pale yellow oil: ^{1}H-NMR ($CDCl_3$) δ 4.11 (s, 3H), 4.04 (s, 3H); ^{19}F-NMR ϕ 149.4 (d, 1F), 149.7 (dd, 1F), 156.9 (d, 1F). Anal. Calcd for $C_8H_6F_3NO_4$: C, 40.51; H, 2.55; N, 5.91. Found: C, 40.45; H, 2.68; N, 5.69.

Method C. Reduction of Dimethoxyfluoronitrobenzenes. The nitro group was reduced by hydrogenation at 40 psi in ethanol/EtOAc over catalytic 10% Pd on carbon. Progress was monitored by TLC. When the reaction was completed (2−4 h), the catalyst was collected on Celite over a glass frit via filtration. The filtrate was concd *in vacuo*, giving pure amines.

1-Amino-2,4-dimethoxy-5-fluorobenzene (23a). Following method C, **22a** (79.2 g, 0.39 mol) gave 67.4 g (100%) of the title compound as a pale gray-brown oil that solidified upon standing: mp 47−49 °C; ^{1}H-NMR ($CDCl_3$) δ 6.53 (s, 1H), 6.50

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(d, J = 5.0, 1H), 3.83 (s, 3H), 3.81 (s, 3H); ^{19}F-NMR ϕ 143.87 (dd, J = 12, 7.0). Anal. Calcd for $C_8H_{10}FNO_2$: C, 56.14; H, 5.89; N, 8.18. Found: C, 56.76; H, 6.04; N, 8.20.

1-Amino-2,4-dimethoxy-3-fluorobenzene (23b). Following method C, **22b** (4.66 g, 23.2 mmol) gave 3.67 g (92%) of the title compound as a pale yellow oil: ^{1}H-NMR ($CDCl_3$) δ 6.52 (t, J = 8.7, 1H), 6.40 (dd, J = 8.8, 2.1, 1H), 3.92 (s, 3H), 3.80 (s, 3H); ^{19}F-NMR ϕ 152.9 (d). Anal. Calcd for $C_8H_{10}FNO_2$: C, 56.14; H, 5.89; N, 8.18. Found: C, 56.14; H, 6.05; N, 8.03.

1-Amino-3,5-difluoro-2,4-dimethoxybenzene (23c). Following method C, **22c** (10.9 g, 49.7 mmol) gave 9.40 g (99.8%) of the title compound as a clear, pale brown oil: ^{1}H-NMR ($CDCl_3$) δ 6.25 (dd, J = 12.1, 2.3, 1H), 3.89 (2 s, 6H), 3.7 (br s, 2H); ^{19}F-NMR ϕ 135.5 (d, 1F), 146.9 (s, 1F). Anal. Calcd for $C_8H_9F_2NO_2$: C, 50.80; H, 4.80; N, 7.40. Found: C, 50.61; H, 4.81; N, 7.26.

The hydrochloride salt was obtained by treating a solution of **22c** with 4 M HCl in dioxane, collecting the precipitate on a Büchner funnel, rinsing with dioxane, and drying *in vacuo* to give the hydrochloride of **22c** as a bone-white powder: mp 213−218 °C dec; ^{1}H-NMR (D_2O) δ 7.1 (br d, 1H), 4.05 (br s, 6H); ^{19}F-NMR ϕ 131.1 (dd, 1F), 141.6 (s, 1F). Anal. Calcd for $C_8H_{10}ClF_2NO_2$: C, 42.59; H, 4.47; N, 6.21. Found: C, 42.75; H, 4.47; N, 6.14.

1-Amino-2,4-dimethoxy-3,5,6-trifluorobenzene (23d). Following method C, **22d** (2.35 g, 9.91 mmol) gave 1.06 g (52%) of the title compound as a pale orange solid. This solid was dissolved in dioxane (8 mL) and treated at room temperature with 4 M HCl in dioxane. After 5 min, the precipitate was collected on a Büchner funnel and rinsed with dioxane (10 mL) followed by drying *in vacuo* over P_2O_5/NaOH to give 0.84 g of the hydrochloride of **23d** as an off-white powder: mp 146−148 °C; ^{1}H-NMR (D_2O) δ 4.08 (s, 3H), 4.00 (s, 3H); ^{19}F-NMR ϕ 149.6 (d, 1F), 154.0 (dd, 1F), 156.8 (d, 1F). Anal. Calcd for $C_8H_9F_3NO_2Cl$: C, 39.44; H, 3.77; N, 5.75. Found: C, 39.79; H, 3.70; N, 5.62. Free base, calcd for $C_8H_8F_3NO_2$: C, 46.39; H, 3.89; N, 6.76. Found: C, 46.49; H, 3.83; N, 6.73.

Method D. Hydrodediazoniation of Aminodimethoxyfluorobenzenes. A solution or mixture of the amine in water/concd HCl (2:1, 0.3 M) was chilled in an ice−salt bath and treated with a cold solution of sodium nitrite (1.05 equiv) in water. The resulting solution was stirred for 15 min, and then H_3PO_2 (50%, 20 equiv) was added over 5 min. The resulting mixture was left at 4 °C overnight, stirred at 20 °C for 2 h, and then diluted with water. The resulting mixture was neutralized with aqueous sodium hydroxide and then extracted with ether (2×). The extract was washed with water (1×) and brine (1×), dried (Na_2SO_4), and concd *in vacuo*. The residue was purified by flash column chromatography, eluting with hexane/EtOAc (95:5).

1,3-Dimethoxy-4-fluorobenzene (24a). Following method D, **23a** (66.81 g, 0.39 mmol) gave 52.81 g (87%) of **24a** as a clear, colorless oil: ^{1}H-NMR ($CDCl_3$) δ 6.97 (dd, J = 11.1, 9.0, 1H), 6.53 (dd, J = 7.0, 2.9, 1H), 6.36 (dt, J = 8.8, 3.0, 1H), 3.86 (s, 3H), 3.77 (s, 3H); ^{19}F-NMR ϕ 146.0 (m). Anal. Calcd for $C_8H_9FO_2$: C, 61.53; H, 5.81. Found: C, 61.26; H, 5.94.

1,3-Dimethoxy-2-fluorobenzene (24b). Following method D, **23b** (1.10 g, 6.43 mmol) gave 0.96 g (96%) of **24b** as a clear, pale yellow liquid: ^{1}H-NMR ($CDCl_3$) δ 6.95 (dt, J = 8.4, 2.2, 1H), 6.59 (t, J = 7.9, 2H), 3.89 (s, 6H); ^{19}F-NMR ϕ 159.2 (s). Anal. Calcd for $C_8H_9O_2F$: C, 61.53; H, 5.81. Found: C, 61.36; H, 5.86.

1,3-Dimethoxy-2,4-difluorobenzene (24c). Following method D, **23c** (0.566 g, 2.99 mmol) gave 0.38 g (73%) of **24c** as a clear, colorless liquid: ^{1}H-NMR ($CDCl_3$) δ 6.80 (td, J = 9.6, 2.5, 1H), 6.58 (m, 1H), 4.00 (s, 3H), 3.87 (s, 3H); ^{19}F-NMR ϕ 138.8 (d, 1F), 150. 1 (d, 1F). Anal. Calcd for $C_8H_8F_2O_2$: C, 55.19; H, 4.63. Found: C, 54.76; H, 4.63.

1,3-Dimethoxy-2,4,5-trifluorobenzene (24d). Following method D, **23d** (0.70 g, 2.9 mmol) gave 0.44 g (80%) of **24d** as a clear, colorless oil: ^{1}H-NMR ($CDCl_3$) δ 6.51 (m, 1H), 4.07 (s, 3H), 3.83 (s, 3H); ^{19}F-NMR ϕ 141.9 (dd, 1F), 156.7 (t, 1F), 163.4 (dd, 1F). Anal. Calcd for $C_8H_7F_3O_2$: C, 50.01; H, 3.67. Found: C, 49.63; H, 3.69.

Method E. Demethylation of Dimethoxyfluorobenzene. A solution of the dimethoxyfluorobenzene in anhydrous CH_2Cl_2 at 20 °C under N_2 was treated with BBr_3 (3.0 equiv, 1.0 M in CH_2Cl_2) via syringe over 5 min. The reaction was monitored by TLC and took 24−48 h to complete; an additional 0.5 equiv of BBr_3 solution was sometimes necessary to drive the reaction to completion. The reaction was carefully quenched with water, and the resulting mixture was stirred until all precipitate(s) dissolved. The resulting solution was extracted with ether (2×). The extract was washed with brine (1×), dried ($MgSO_4$), and concd to fluorinated resorcinols. The crude fluororesorcinols were purified by sublimation (60−100 °C at 0.3−0.5 Torr).

4-Fluororesorcinol (25a). Following method E, **24a** (52.8 g, 0.34 mol) gave 40.5 g (95%) of **25a** as a colorless crystalline solid: mp 94−96 °C; ^{1}H-NMR (DMSO-d_6) δ 8.39 (br, 1H), 8.00 (br, 1H), 6.89 (dd, J = 10.1, 9.0, 1H), 6.48 (dd, J = 7.3, 3.0, 1H), 6.27 (m, 1H); ^{19}F-NMR ϕ 145.82 (m). Anal. Calcd for $C_6H_5FO_2$: C, 56.26; H, 3.93. Found: C, 56.23; H, 3.93.

2-Fluororesorcinol (25b). Following method E, **24b** (0.96 g, 6.1 mmol) gave 0.75 g (95%) of **25b** as a colorless crystalline solid: mp 114−116 °C; ^{1}H-NMR ($CDCl_3$) δ 8.52 (br s, 2H), 6.58 (m, 1H), 6.30 (t, J = 7.9, 2H); ^{19}F-NMR ϕ 162.3 (t). Anal. Calcd for $C_6H_5FO_2$: C, 56.26; H, 3.93. Found: C, 56.05; H, 3.96.

2,4-Difluororesorcinol (25c). Following method E, **24c** (0.64 g, 3.7 mmol) gave 0.49 g (90%) of **25c** as a colorless crystalline solid: mp 99−100 °C; ^{1}H-NMR ($CDCl_3$) δ 8.99 (s, 1H), 8.62 (s, 1H), 6.32 (td, J = 9.1, 2.2, 1H), 6.05 (m, 1H); ^{19}F-NMR ϕ 145.4 (m, 1F), 156.0 (m, 1F). Anal. Calcd for $C_6H_4F_2O_2$: C, 49.33; H, 2.76. Found: C, 48.96; H, 2.80.

2,4,5-Trifluororesorcinol (25d). Following method E, **24d** (0.42 g, 2.2 mmol) gave 0.36 g (100%) of **25d** as a colorless crystalline solid: mp 69−71 °C; ^{1}H-NMR ($CDCl_3$) δ 6.05 (m); ^{19}F-NMR ϕ 144.4 (s, 1F), 162.2 (s, 1F), 169.7 (s, 1F). Anal. Calcd for $C_6H_3F_3O_2 \cdot H_2O$: C, 39.58; H, 2.77. Found: C, 39.58; H, 2.65.

5-Fluororesorcinol. Following method E, 3,5-dimethoxyfluorobenzene (5.0 g, 32.0 mmol) gave 3.69 g (92%) of 5-fluororesorcinol as a colorless crystalline solid: mp 134−135.5 °C; ^{1}H-NMR (DMSO-d_6) δ 9.60 (s, 2H), 6.05 (s, 1H), 5.97 (d, 2H); ^{19}F-NMR ϕ 108.26 (t, J = 11.1). Anal. Calcd for $C_6H_5FO_2$: C, 56.26; H, 3.93. Found: C, 56.33; H, 3.98.

Method F. Preparation of Fluoresceins. Trimellitic anhydride (**26**), phthalic anhydride (**27**), or tetrafluorophthalic anhydride (**28**) (1 equiv) was added to a solution of the appropriate fluorinated resorcinol (2 equiv) in methanesulfonic acid (1 M). The resulting mixture was heated under dry nitrogen at 80−85 °C for 36−48 h. The cooled mixture was poured into 7 volumes of ice water followed by filtration. The filtrand, containing the fluorinated fluorescein, was dried at 60 °C *in vacuo* to constant weight.

Method G. Purification of Fluoresceins. The dried fluorescein was converted to the diacetate by dissolution in acetic anhydride and pyridine and heating briefly, and the resulting solution was subjected to aqueous workup and recrystallization from absolute ethanol. The diacetate was dissolved in THF/MeOH (1:1) to yield a 5% (w/v) solution and stirred with NH_4OH (10 equiv) for 2 h. The reaction mixture was poured into 5 volumes of cold water, acidified to pH 2 with 10% HCl, and concd *in vacuo* to remove THF/MeOH. The solid was collected by filtration, washed with cold water, and dried *in vacuo* at 60 °C to constant weight (Scheme 4).

Method H. Purification of Fluoresceins. Similar to method G except that the recrystallization step was replaced with silica gel flash column chromatography eluting with $CHCl_3$/MeOH (95:5).

5(6)-Carboxy-2′,7′-difluorofluorescein (14a,b). Following method F, **25a** (10.0 g, 78.0 mmol) and **26** (7.60 g, 39.6 mmol) gave 14.80 g (92%) of the title compound as an orange powder.

Isolation of 14bAc$_2$. The solid thus obtained in the above preparation was heated with Ac_2O (35.0 g, 340 mmol) and pyridine (6.09 g, 77 mmol) for 5 min at 80 °C. The solution was cooled down and then placed in a freezer for 16 h. The precipitate was collected, washed with Ac_2O (2 × 5 mL) and ether (2 × 10 mL), and pumped in a desiccator with P_2O_5 for 12 h to yield 6.25 g (34%) of 6-carboxy-2′,7′-difluorofluorescein diacetate, pyridinium salt: ^{1}H-NMR ($CDCl_3$) δ 8.67 (m, 2H),

8.41 (d, J = 8.1, 1H), 8.12 (d, J = 8.1, 1H), 7.91 (s, 1H), 7.84 (m, 1H), 7.42 (m, 2H), 7.15 (d, J = 6.1, 2H), 6.59 (d, J = 9.7, 2H), 2.34 (s, 6H); ^{19}F-NMR ϕ 131.73 (dd, J = 10.1, 8.7). Anal. Calcd for $C_{30}H_{19}F_2NO_9$: C, 62.61; H, 3.33; N, 2.43. Found: C, 62.01; H, 3.25; N, 2.37.

Isolation of 14a Diacetate. The filtrate from the above preparation was poured into 120 mL of water, stirred for 30 min, and extracted with EtOAc (50 mL × 3). The combined extract was washed with 3% HCl solution (40 mL) and brine (40 mL) and concd *in vacuo* to yield 12.5 g of a pale brown oil. The oil was recrystallized from CH_2Cl_2 to yield 5.1 g (28%) of 5-carboxy-2′,7′-difluorofluorescein diacetate as colorless crystals: ^{1}H-NMR ($CDCl_3$) δ 8.79 (s, 1H), 8.46 (d, J = 8.1, 1H), 7.34 (d, J = 8.1, 1H), 7.17 (d, J = 6.2, 2H), 6.60 (d, J = 9.7, 2H), 2.35 (s, 6H); ^{19}F-NMR ϕ 131.44 (br). Anal. Calcd for $C_{25}H_{14}F_2O_9 \cdot 0.5CH_2Cl_2$: C, 56.84; H, 2.80. Found: C, 56.60; H, 2.81.

More isomerically pure material could be isolated from the mother liquor by alternate recrystallization of **14b**Ac_2, pyridinium salt from toluene/pyridine and **14a**Ac_2 from CH_2Cl_2.

5-Carboxy-2′,7′-difluorofluorescein (14a). **14a**Ac_2 (1.00 g, 2.01 mmol) was dissolved in 15 mL of THF and diluted with 15 mL of MeOH and 3 mL of water. To the solution was added 4 mL of NH_4OH (28%), and the mixture was stirred for 2 h. The reaction mixture was filtered and diluted with 60 mL of water. The filtrate was acidified with 10% HCl to pH 2 and concd *in vacuo* to remove the THF and MeOH. The resulting precipitate was collected, washed with cold water (3 × 20 mL), and pumped in a desiccator with P_2O_5 at 60 °C for 24 h to yield 0.68 g (83%) of an orange solid: ^{1}H-NMR (DMSO-d_6) δ 8.39 (s, 1H), 8.29 (d, J = 8.0, 1H), 7.40 (d, J = 8.0, 1H), 6.88 (d, J = 7.6, 2H), 6.63 (d, J = 11.3, 2H); ^{19}F-NMR ϕ 135.24 (t, J = 8.5). Anal. Calcd for $C_{21}H_{10}F_2O_7 \cdot 0.5H_2O$: C, 59.86; H, 2.63. Found: C, 59.57; H, 2.53.

6-Carboxy-2′,7′-difluorofluorescein (14b). Starting from 6-carboxy-2′,7′-difluorofluorescein diacetate and using a similar procedure as described above, **14b** was obtained in 92% yield: ^{1}H-NMR (DMSO-d_6) δ 8.25 (d, 1H), 8.10 (d, 1H), 7.70 (s, 1H), 6.89 (d, 2H), 6.58 (d, 2H); ^{19}F-NMR ϕ 135.20 (dd). Anal. Calcd for $C_{21}H_{10}F_2O_7 \cdot 0.5H_2O$: C, 59.86; H, 2.63. Found: C, 59.53; H, 2.49.

5(6)-Carboxy-4′,5′-difluorofluorescein (15a,b). Following method F, **25b** (0.35 g, 2.7 mmol) and **26** (0.36 g, 1.4 mmol) gave 0.42 g (82%) of the title compound as a reddish-brown powder: R_f ($CHCl_3$/MeOH/AcOH, 20:4:1) 0.28; UV max (pH 8.5 phosphate buffer) 511 nm (ϵ 67 000); emission max 534 nm. Anal. Calcd for $C_{21}H_{10}F_2O_7 \cdot 1.1H_2O$: C, 58.62; H, 2.87. Found: C, 58.00; H, 2.88.

The diacetate was prepared as a nonfluorescent reddish-orange powder: R_f ($CHCl_3$/MeOH/AcOH, 20:4:1) 0.54; ^{1}H-NMR ($CDCl_3$) δ 8.4 (m, 1H), 8.1 (m, 1H), 7.8 (m, 1H), 6.9 (m, 2H), 6.6 (m, 2H), 2.4 (m, 6H); ^{19}F-NMR ϕ 145.9 (dd, 1F), 146.2 (m). Anal. Calcd for $C_{21}H_{10}F_2O_7 \cdot 2H_2O$: C, 56.26; H, 3.15. Found: C, 56.92; H, 3.19.

5(6)-Carboxy-2′,4′,5′,7′-tetrafluorofluorescein (16a,b). Following methods F and H, **25c** (0.65 g, 4.3 mmol) and **26** (0.57 g, 2.2 mmol) gave 0.57 g (60%) of the title compound as a brown powder: R_f ($CHCl_3$/MeOH/AcOH, 20:4:1) 0.22.

The diacetate was prepared as a nonfluorescent reddish-orange powder: R_f ($CHCl_3$/MeOH/AcOH, 20:4:1) 0.50; ^{1}H-NMR (DMSO-d_6) δ 8.4−7.6 (m, 3H), 7.05 (2 dd, 2H), 2.4 (2 s, 6H); ^{19}F-NMR ϕ 125.6 (d), 125.7 (d), 140.0 (s), 140.1 (s). Anal. Calcd for $C_{21}H_8F_4O_7 \cdot 0.9H_2O$: C, 54.30; H, 2.12. Found: C, 54.58; H, 2.31.

2′,7′-Difluorofluorescein (17). Following methods F and G, **25a** (0.70 g, 5.5 mmol) and phthalic anhydride (0.41 g, 2.8 mmol) gave 0.85 g (85%) of **17** as an orange powder: ^{1}H-NMR (DMSO-d_6) δ .7.99 (d, J = 7.5, 1H), 7.80 (t, J = 7.4, 1H), 7.73 (t, J = 7.3, 1H), 7.29 (d, J = 7.5, 1H), 6.88 (d, J = 7.4, 2H), 6.48 (d, J = 11.2, 2H); ^{19}F-NMR ϕ 134.35 (dd, J = 7.3, 11.3). Anal. Calcd for $C_{20}H_{10}F_2O_5 \cdot 0.2H_2O$: C, 64.59; H, 2.81. Found: C, 64.40; H, 2.83.

4,5,6,7-Tetrafluorofluorescein (18). Following methods F and G, resorcinol (22.02 g, 0.20 mol) and **28** (22.10 g, 0.10 mol) gave 32.56 g (74%) of **18** as a brick-red powder: ^{1}H-NMR (DMSO-d_6) δ 7.00 (d, J = 8.7, 2H), 6.70 (d, J = 2.2, 2H), 6.60 (dd, J = 8.7, 2.3, 2H): ^{19}F-NMR ϕ 135.70 (m, 1F), 139.70 (m, 2F), 147.59 (t, 1F). Anal. Calcd for $C_{20}H_8F_4O_5$: C, 59.42; H, 1.99. Found: C, 59.39; H, 2.01.

18Ac$_2$: ^{1}H-NMR ($CDCl_3$) δ 7.15 (s, 2H), 6.99 (d, 2H), 6.92 (d, 2H), 2.31 (s, 6H); ^{19}F-NMR ϕ 137.26 (m, 1F), 140.79 (m, 1F), 141.21 (m, 1F), 148.97 (m, 1F). Anal. Calcd for $C_{24}H_{12}F_4O_7$: C, 59.03; H, 2.48. Found: C, 59.00; H, 2.69.

2′,4,5,6,7,7′-Hexafluorofluorescein (19). Following methods F and G, **25a** (1.01 g, 7.9 mmol) and **28** (1.91 g, 8.7 mmol) gave 2.85 g (86%) of **19** as an orange powder: ^{1}H-NMR (DMSO-d_6) δ 7.11 (d, J = 11.3, 2H), 6.88 (d, J = 7.6, 2H); ^{19}F-NMR ϕ 134.73 (br, 2F), 135.29 (m, 1F), 139.96 (br, 2F), 148.01 (t, J = 18.9, 1F).

19Ac$_2$: ^{1}H-NMR ($CDCl_3$) δ 7.17 (d, J = 6.3, 2H), 6.76 (d, J = 9.6, 2H), 2.36 (s, 6H); ^{19}F-NMR ϕ 130.88 (t, J = 8.5, 2F), 136.09 (m, 1F), 139.74 (m, 1F), 141.01 (t, J = 20.7, 1F), 147.77 (t, J = 17.8, 1F). Anal. Calcd for $C_{20}H_6F_6O_5 \cdot H_2O$: C, 52.42; H, 1.75. Found: C, 52.69; H, 1.92.

2′,4,4′,5,5′,6,7,7′-Octafluorofluorescein (20). Following methods F and H, **25c** (0.28 g, 2.0 mmol) and **28** (0.22 g, 1.0 mmol) gave 0.31 g (65%) of **20** as a dark brown powder: ^{1}H-NMR (DMSO-d_6) δ 7.11 (d, J = 9.5); ^{19}F-NMR ϕ 130.49 (t, J = 10.2, 2F), 134.86 (m, 1F), 139.11 (m, 1F), 139.64 (t, J = 19.6, 1F), 147.46 (t, J = 20.2, 1F), 148.25 (d, J = 9.3, 2F). Anal. Calcd for $C_{20}H_4F_8O_5 \cdot 0.5H_2O$: C, 49.50; H, 1.03. Found: C, 49.42; H, 1.13.

20Ac$_2$: ^{1}H-NMR (DMSO-d_6) δ 7.53 (d, J = 11.0, 2H), 2.47 (s, 6H); ^{19}F-NMR ϕ 124.69 (d, J = 10.2, 2F), 134.36 (m, 1F), 137.81 (m, 1F), 138.82 (t, J = 20.0, 1F), 139.11 (s, 2F), 146.79 (t, J = 19.9, 1F). Anal. Calcd for $C_{24}H_8F_8O_7$: C, 51.45; H, 1.44. Found: C, 51.07; H, 1.67.

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