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# Creation of Superior Carboxyfluorescein Dyes by Blocking Donor-Excited Photoinduced Electron Transfer

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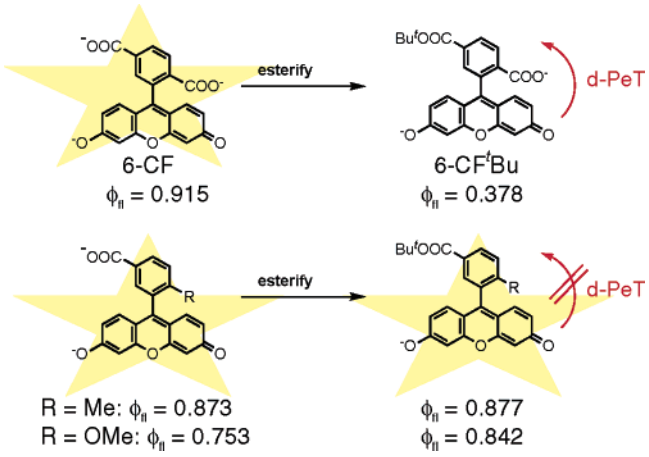
## ABSTRACT




**Carboxyfluoresceins are widely utilized as fluorescence labeling reagents, but we recently found that their emission intensity is markedly decreased after esterification. On the basis of our hypothesis that the fluorescence decrease is due to a donor-excited photoinduced electron transfer (d-PeT) process, we have developed novel carboxyfluorescein derivatives in which the d-PeT process is hampered, and the emission intensity is not decreased upon esterification. These novel dye derivatives display high quantum yields and are expected to be useful as labeling agents.**

In the field of chemical biology, it is often required to detect biological substances, such as proteins, DNAs, and sugars, with high sensitivity and selectivity. Fluorescence labeling reagents enable the detection of the target biological molecules through covalent binding.[1−4] Organic compound based fluorescent dyes are widely used because of their small molecular weight and convenience in use, and the specific detection is based on their characteristic excitation and emission properties. Fluorescein[5−9] has a relatively high

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(1) Scherrer, P.; Alexiev, U.; Marti, T.; Khorana, H. G.; Heyn, M. P. *Biochemistry* **1994**, *33*, 13684−13692.
(2) Alexiev, U.; Marti, T.; Heyn, M. P.; Khorana, H. G.; Scherrer, P. *Biochemistry* **1994**, *33*, 13693−13699.
(3) Maeda, H.; Ishida, N.; Kawauchi, H.; Tuzimura, K. *J. Biochem.* **1969**, *65*, 777−783.
(4) Hegyvary, C.; Jorgensen, P. L. *J. Biol. Chem.* **1981**, *256*, 6294−6303.
(5) Baeyer, A. *Ber. Dtsch. Chem. Ges.* **1871**, *4*, 555−558.
(6) Orndorff, W. R.; Hemmer, A. J. *J. Am. Chem. Soc.* **1927**, *49*, 1272−1280.

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fluorescence quantum yield ($\phi_{fl}$) of 0.85 with the absorption maximum ($Abs_{max}$) of 492 nm and the emission maximum ($Em_{max}$) of 511 nm. Prerequisites for fluorescence labeling reagents are to bear an intensely emitting fluorophore and a reactive functional group to bind to the target molecule. From these viewpoints, carboxyfluoresceins with fluorescence properties similar to fluorescein are very useful as fluorescence labeling reagents and have indeed been widely utilized for biological applications,[10−15] although in most cases a mixture of 5- and 6-carboxylate isomers is used due to the difficulty of separation.[16,17]

Recently, we found that the emission intensity of carboxyfluoresceins declines markedly upon esterification of the carboxyl functional groups. For example, *t*-butyl esterification of 6-carboxyfluorescein (6-CF) decreased the $\phi_{fl}$ value from 0.915 to 0.378 at pH 7.4, and the corresponding change for 5-carboxyfluorescein (5-CF) was from 0.834 to 0.694 (Figure 1). This suppression of fluorescence emission

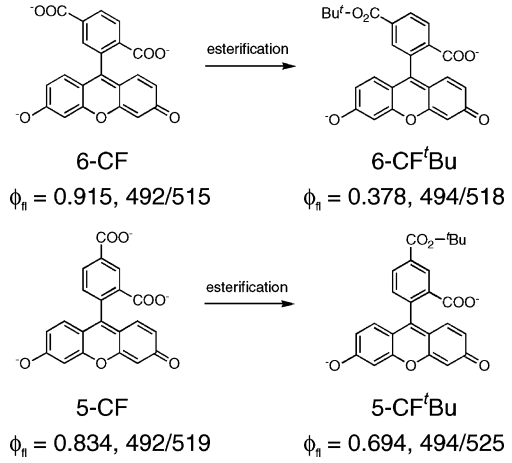



**Figure 1.** Fluorescence quantum yields ($\phi_{fl}$) and absorption/emission maxima (nm) of carboxyfluoresceins and their *t*-butyl esters in 0.1 M sodium phosphate buffer (pH 7.4).

may be rationally explained by the occurrence of a donor-excited photoinduced electron transfer (d-PeT) process from the xanthene unit to the benzene unit[18−20] because there is no major shift in $Abs_{max}$/$Em_{max}$ accompanying the reduction of the $\phi_{fl}$ values (Figure 1). This hypothesis is also supported by our previous findings on modulation of fluorescence properties via the d-PeT process: the $\phi_{fl}$ value of tricarboxyfluorescein (0.817) is far higher than that of its trimethyl ester derivative (0.001).[21] We therefore aimed to design and synthesize novel fluorescence labeling reagents in which the d-PeT process is hampered and the emission intensity is maintained even after labeling events.

The process of d-PeT involves transfer of one electron from the excited xanthene unit to the lowest unoccupied molecular orbital (LUMO) of the electron-deficient benzene unit, leading to fluorescence quenching (Figure 2A). Thus,

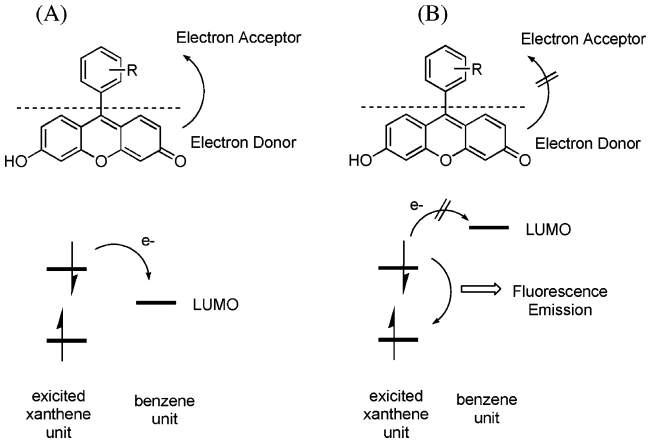



**Figure 2.** Electron flow involved in d-PeT and molecular orbital diagrams. (A) Fluorescence emission is quenched by d-PeT. (B) Fluorescence emission is restored by preventing d-PeT.

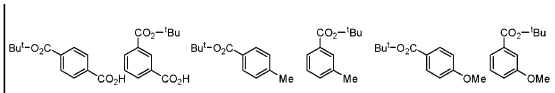
elevation of the LUMO level by raising the electron density of the benzene unit should prevent the d-PeT process and result in a high quantum yield of the compound (Figure 2B).

The ease of electron transfer from the electron donor to the acceptor is predicted by the Rehm−Weller equation[21,22] and is correlated with the LUMO energy level.

The LUMO levels of the acceptor units of 6-CF$^t$Bu and 5-CF$^t$Bu, terephthalic acid mono *t*-butyl ester (**1**) and isophthalic acid mono *t*-butyl ester (**2**), were obtained by means of B3LYP/6-31G calculations. The small quantum yields of 6-CF$^t$Bu and 5-CF$^t$Bu can be explained by the low LUMO levels of their acceptor units **1** and **2**, as shown in Table 1, and this is also supported by our recent findings regarding fluorescence quenching owing to the electron-deficient benzene moiety of the acceptor unit.[21] The LUMO levels in Table 1 were calculated for the neutral forms, as the electron deficiency of the benzoate derivatives of 6-CF

(7) Weber, G.; Teale, F. W. J. *Trans. Faraday Soc.* **1958**, *54*, 640−648.
(8) Jiao, G.-S.; Han, J. W.; Burgess, K. *J. Org. Chem.* **2003**, *68*, 8264−8267.
(9) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888−4894.
(10) Tobey, S. L.; Anslyn, E. V. *Org. Lett.* **2003**, *5*, 2029−2031.
(11) Paradiso, A. M.; Tsien, R. Y.; Machen, T. E. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7436−7440.
(12) Souto, A. A.; Acuña, A. U.; Andreu, J. M.; Barasoain, I.; Abal, M.; Amat-Guerri, F. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2710−2712.
(13) Setsukinai, K.; Urano, Y.; Kakinuma, K.; Majima, H. J. *J. Biol. Chem.* **2003**, *278*, 3170−3175.
(14) Takakusa, H.; Kikuchi, K.; Urano, Y.; Sakamoto, S.; Yamaguchi, K.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 1653−1657.
(15) Kojima, H.; Nanatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446−2453.
(16) Rossi, F. M.; Kao, J. P. Y. *Bioconjugate Chem.* **1997**, *8*, 495−497.
(17) Ueno, Y.; Guan-Sheng, J.; Kevin, B. *Synthesis* **2004**, 2591−2593.
(18) Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, *125*, 8666−8671.
(19) Kavarnos, G. J.; Turro, N. J. *Chem. Rev.* **1986**, *86*, 401−449.
(20) Zhang, H.; Zhou, Y.; Zhang, M.; Shen, T.; Li, Y.; Zhu, D. *J. Phys. Chem. B* **2002**, *106*, 9597−9603.
(21) Ueno, T.; Urano, Y.; Setsukinai, K.; Takakusa, H.; Kojima, H.; Kikuchi, K.; Ohkubo, K.; Fukuzumi, S.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 14079−14085.
(22) Rehm, D.; Weller, A. *Isr. J. Chem.* **1970**, *8*, 259−271.

**Table 1.** LUMO Energy Levels of the Electron-Acceptor Portions by B3LYP/6-31G Calculations

| Acceptor Portions |  | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** |
| Calculated LUMO Levels (hartrees) | -0.0810 | -0.0669 | -0.0436 | -0.0456 | -0.0392 | -0.0453 |

and 5-CF could not be calcualted. We also measured the reduction potential of terephthalic acid mono *t*-butyl ester (**1**), the benzene moiety of the less fluorescent derivative 6-CF$^t$Bu, and found that the value was in the range appropriate for d-PeT (Figure S1, provided in the Supporting Information), as discussed in our previous paper.[21] Thus, both lines of evidence indicate that the small quantum yield of 6-CF$^t$Bu can be explained in terms of the low LUMO level of the acceptor unit **1**.

We have recently discovered that replacement of the carboxyl group at the 2 position of fluorescein with other groups does not diminish the intensity of fluorescence. The compounds bearing a methyl or methoxy group at the 2 position, termed 2-Me TokyoGreen and 2-OMe TokyoGreen, respectively, exhibit $\phi_{fl}$ values as high as that of fluorescein.[9,23] We thus conducted B3LYP/6-31G calculations of the *t*-butyl esters **3**−**6**, possessing methyl or methoxy groups in place of carboxylates. Introduction of electron-donating methyl and methoxy groups appeared to result in higher LUMO values of **3**−**6**, as predicted. Considering the xanthene unit as the electron donor and the benzene unit as the electron acceptor, we have newly designed carboxyfluorescein dyes bearing methyl and methoxy substituents, **7**−**10** (Figure 3). On the basis of our hypothesis that a high LUMO

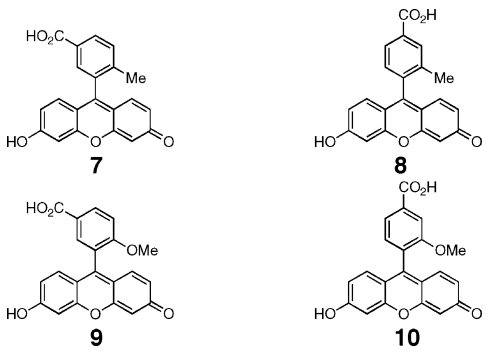



**Figure 3.** Structures of the proposed carboxyfluorescein dyes, **7**−**10**.

level suppresses d-PeT, these carboxyfluorescein compounds **7**−**10** were expected to display higher $\phi_{fl}$ values than 5-CF$^t$Bu and 6-CF$^t$Bu after esterification.

The synthesis of the designed compounds began with bromomethylbenzoic and bromomethoxybenzoic acids. 3-Bromo-4-methylbenzoic acid (**11**) was converted to the *t*-butyl ester **12** (Scheme S1, provided in the Supporting Information), which was treated with 3,6-bis(*tert*-butyldimethylsilyloxy)-9*H*-xanthen-9-one[24,25] in the presence of *t*-BuLi, followed by deprotection of the dimethylsilyloxy groups and condensation in 2 N HCl solution. Cleavage of the *t*-butyl group of the resultant compound **13** with TFA/$CH_2Cl_2$ (1:1) furnished the free carboxyl acid **7**. Scheme S2 (provided in the Supporting Information) shows the preparation of compound **8**,[23] and Scheme S3 (provided in the Supporting Information) shows that of compound **9**. Both of them were synthesized in good yields using steps and reagents similar to those in Scheme S1. After Sandmeyer-like reaction from **20** to **21**, compound **21** had to be protected as the pyrrolidine amide **22** for the succeeding reactions (Scheme S4, provided in the Supporting Information). The amide intermediate **23** was deprotected with 1 N NaOH to yield the carboxylic acid **10**, which was then finally transformed to the corresponding *t*-butyl ester **24**. The identities of the four compounds and their *t*-butyl esters were confirmed by spectrometric characterization in advance of further analysis of the fluorescence properties.

The fluorescence of the dyes **7**−**10** and their *t*-butyl esters **13**, **16**, **19**, and **24** and the amide **23** (Figure 4) was

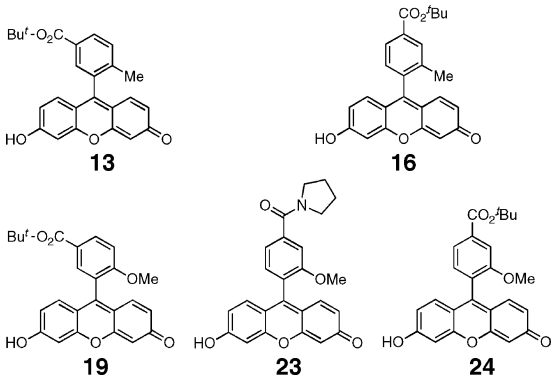



**Figure 4.** Structures of the *t*-butyl esters **13**, **16**, **19**, and **24** and the amide **23**.

characterized. In view of the equilibrium constant of the xanthene moiety (p$K_a$ = 6.2), the spectra were recorded in sodium phosphate buffer of pH 7.4. The $Abs_{max}$, the fluorescence $Em_{max}$ excited at 490 nm, and the $\phi_{fl}$ values are summarized in Table 2. All the spectra displayed $Abs_{max}$ values of around 490 nm, and the newly synthesized *t*-butyl esters were found to possess high quantum yields, as predicted. Among them, compound **13**, the *t*-butyl ester of 5-carboxy-2-Me TokyoGreen (**7**), displayed the most prominent fluorescence intensity in contrast to the *t*-butyl ester of

(23) Mottram, L. F.; Boonyarattanakalin, S.; Kovel, R. E.; Peterson, B. R. *Org. Lett.* **2006**, *8*, 581−584.
(24) Grover, P. K.; Shah, G. D.; Shah, R. C. *J. Chem. Soc.* **1955**, 3982−3985.
(25) Kenmoku, S.; Urano, Y.; Kanda, K.; Kojima, H.; Kikuchi, K.; Nagano, T. *Tetrahedron* **2004**, *60*, 11067−11073.

**Table 2.** Fluorescence Properties of the Newly Synthesized Carboxyfluorescein Dyes and Their *t*-Butyl Esters in 0.1 M Sodium Phosphate Buffer (pH 7.4)

| | pH 7.4 | | |
|---|---|---|---|
| | $Abs_{max}$ | $Em_{max}$[a] | $\phi_{fl}$ value[b] |
| 6-CF | 492 | 515 | 0.915 |
| 5-CF | 492 | 519 | 0.834 |
| 6-CF$^t$Bu | 494 | 518 | 0.378 |
| 5-CF$^t$Bu | 494 | 525 | 0.694 |
| **7** | 493 | 512 | 0.873 |
| **8** | 493 | 514 | 0.830 |
| **9** | 495 | 518 | 0.753 |
| **10** | 496 | 523 | 0.776 |
| **13** | 494 | 514 | 0.877 |
| **16** | 495 | 517 | 0.771 |
| **19** | 497 | 519 | 0.842 |
| **23** | 497 | 521 | 0.740 |
| **24** | 498 | 527 | 0.658 |

[a] Excited at 490 nm. [b] Obtained by calculation, based on fluorescein as the standard ($\phi_{fl} = 0.850$).

6-CF, as graphically shown in Figure 5. Compounds **13** and **19**, whose carboxyl esters are para to the methyl or methoxy group, exhibited remarkable recovery of the quantum yields ($\phi_{fl} = 0.877$ for **13** and $\phi_{fl} = 0.842$ for **19**), compared with 6-CF$^t$Bu ($\phi_{fl} = 0.378$) (Table 2). The above-mentioned para-substituted compounds **13** and **19** have higher values of quantum yield than the meta-positioned esters **16** and **24**. A plausible explanation is that the electron-donating character of the para-situated methyl and methoxy groups is greater than that of meta-situated ones owing to resonance effects, and this is supported by the higher LUMO values of the para-substituted acceptors (Table 1). The amide derivative **23** also showed a high quantum yield ($\phi_{fl} = 0.740$).

On the basis of the rationale of the donor-derived fluorescence quenching mechanism, we have designed and synthesized novel carboxyfluorescein dyes in which the d-PeT process is hampered and full fluorescence is maintained, taking *t*-butyl esters as a model. Replacement of the carboxylate with electron-donating methyl or methoxy substituents successfully blocked the d-PeT process and provided the fully emitting esters **13** and **19**. These novel carboxyfluorescein dyes should be useful for more sensitive fluorescence labeling, especially for hydroxyl-containing compounds such as steroids, than is possible with currently available labeling reagents. Further, the synthetic route presented herein enabled us to acquire a single isomer of each derivative, whereas the known method generates a mixture of 5-CF and 6-CF. These novel carboxyfluorescein dyes and the rationale introduced here provide a solid foundation for the development of superior fluorescence labeling agents for chemical-biological research.

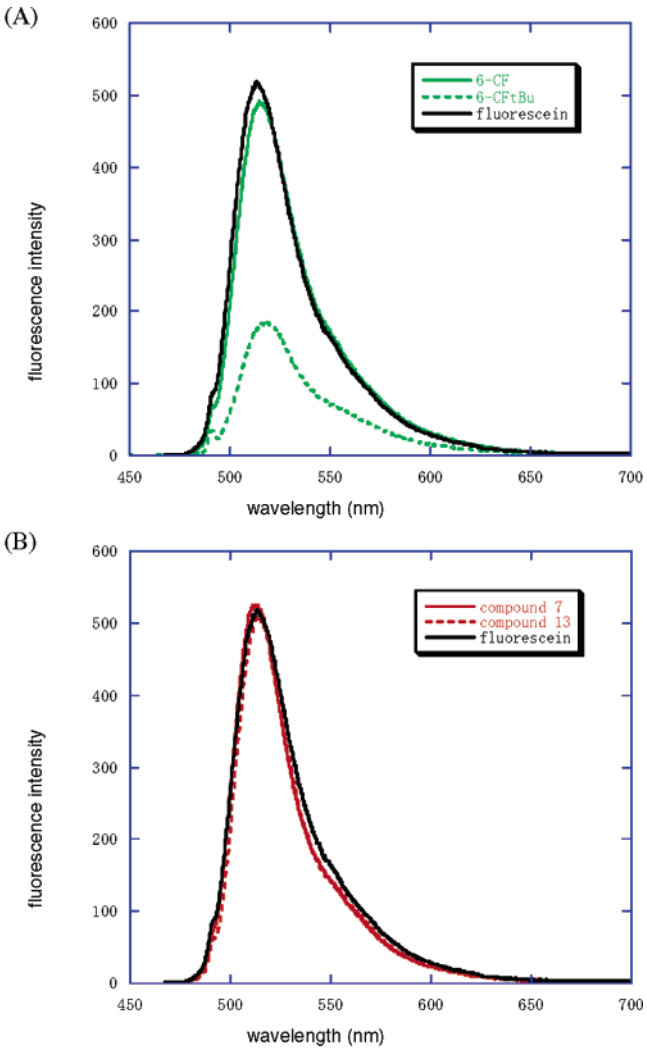



**Figure 5.** Fluorescence emission spectra (excited at 490 nm) in 0.1 M sodium phosphate buffer (pH 7.4). (A) 6-CF and its *t*-butyl ester, 6-CF$^t$Bu. (B) Compound **7** and its *t*-butyl ester, compound **13**.

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**Supporting Information Available:** Supporting experimental details for the preparation and characterization of compounds **1**, **7**−**10**, **12**, **13**, **15**, **16**, **18**, **19**, and **21**−**24** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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