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Effect of Binding and Conformation on Fluorescence Quenching in New 2′,7′-Dichlorofluorescein Derivatives

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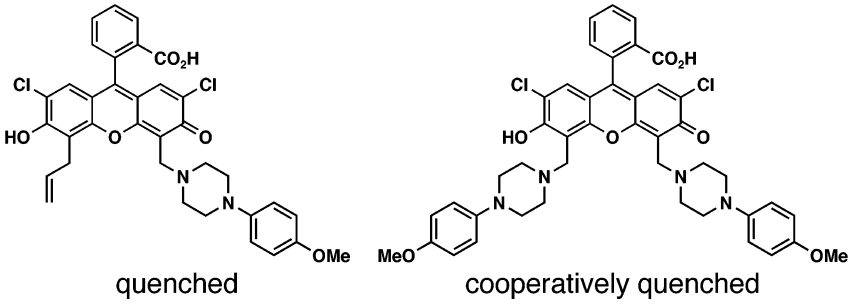
ABSTRACT



Symmetrical and unsymmetrical 2′,7′-dichlorofluorescein (DCF) derivatives have been synthesized by means of Mannich reactions and an aromatic Claisen rearrangement. NMR and fluorescence spectroscopic studies reveal the correlation between the conformations, the photoinduced electron transfer mechanism, and fluorescent intensities of these DCF derivatives. Two quenching nitrogen atoms cooperatively and reversibly suppress the fluorescence of the chromophore.

Fluorescent small molecules have become indispensable tools in biology for imaging biomolecules.[1−4] The binding of an analyte to a fluorescent sensor can change the light emission spectrum and/or change the intensity of the emitted light. A useful fluorogenic sensor system should maximize the difference between the fluorescent intensity of a free sensor and that of the sensor bound to the analyte. To achieve a desirable signal-to-background ratio, it is therefore important to suppress the fluorescent intensity of an unbound fluorophore.[5]

To initiate our program in sensor development, we chose to use 2′,7′-dichlorofluorescein (DCF) as the fluorophore core. The fluorescence of DCF is nearly pH-independent under physiological conditions,[3] and many fluorescein derivatives have been used to image biomolecules.[4,6,7]

We reasoned that aniline side chains on this core would act as switches and reversibly quench the fluorescence emission of the xanthene in the physiological pH range because of their low pK_a values and efficient electron-transfer ability. We designed compounds **1**−**4** (Scheme 1) to investigate the effect of aniline nitrogen atoms in the photoinduced electron transfer (PET) mechanism with respect to the nitrogen's electron density and proximity to the xanthene.[8]

(1) Mason, W. T. *Fluorescent and Luminescent Probes for Biological Activity*, 2nd ed.; Academic Press: New York, 1999.

(2) (a) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440−3450. (b) Pearce, D. A.; Jotterand, N.; Carrico, I. S.; Imperiali, B. *J. Am. Chem. Soc.* **2001**, *123*, 5160−5161.

(3) Walkup, G. K.; Burdette, S. C.; Lippard, S. J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2000**, *122*, 5644−5645.

(4) Hirano, T.; Kikuchi, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 6555−6562.

(5) (a) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566. (b) Martínez-Máñez, R.; Sancenón, F. *Chem. Rev.* **2003**, *103*, 4419−4476.

(6) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269−272.

(7) Burdette, S. C.; Walkup, G. K.; Spingler, B.; Tsien, R. Y.; Lippard, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 7831−7841.

(8) Mannich product between DCF, formaldehyde, and 1-(4-chlorophenyl)piperazine could not be isolated due to its instability.

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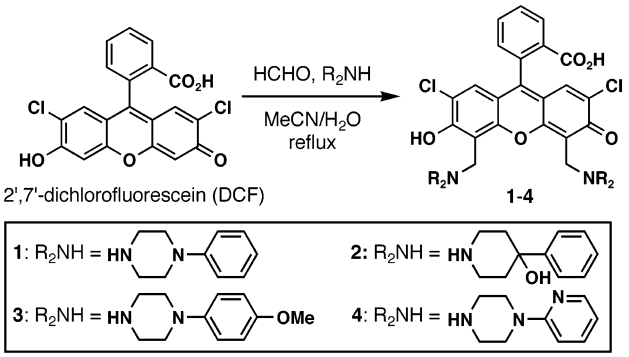
Scheme 1. Mannich Reactions of DCF



The commercially available secondary amines (Scheme 1, box) were coupled with DCF in the presence of formaldehyde by means of Mannich reactions.[3] The isolated yields of the corresponding products **1**−**4** were 75−85%.

We measured the relative quantum yields of compounds **1**−**4** (Table 1). Compound **1** showed a low quantum yield

Table 1. Quantum Yields of Compounds **1**−**4** and **10**

compound	quantum yield[a]
1	6.6%
2	89%
3	3.6%
4	21%
10	20%

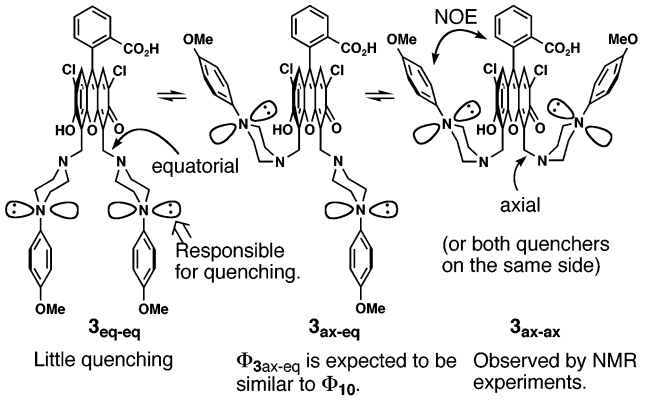
[a] Relative to DCF.

(6.6% of Φ_{DCF}; Φ_{DCF} = quantum yield of DCF), suggesting that if a suitable method for unquenching can be found, this compound or its congeners can be used as fluorogenic compounds for optical visualization. Compound **2**, lacking aniline nitrogen atoms, showed a quantum yield close to that of DCF (89% of Φ_{DCF}). These results are consistent with the widely accepted PET quenching mechanism based on coupling of the electrons of the aniline system to the relaxation of the excited chromophore. On the basis of this mechanism, we hypothesized that *para*-methoxyaniline derivative **3** should have a lower quantum yield due to the higher HOMO level of the aniline system. The quantum yield of **3** was found to be 3.6% of Φ_{DCF}. This result implies that compound **3** has great potential as a sensitive fluorogenic compound, the fluorescence signal of which can be increased dramatically at ∼525 nm if the quenching mechanism is interrupted.

To further verify that the electron densities of the aniline nitrogen atoms of DCF derivatives **1**−**3** are correlated with their quantum yields by the PET mechanism, we measured the quantum yield of **4**. Compound **4** fluoresced much more intensely than **1** (21% of Φ_{DCF}), which is consistent with the PET mechanism because the HOMO level of the aniline nitrogen atoms of **4** is lower than that of **1** due to the electron-withdrawing nature of the pyridine ring.[9]

These experiments demonstrate a correlation between the HOMO level of the aniline nitrogen atom and the quantum yield of the DCF derivatives. We were also interested in the effect of structure and conformation on the quenching phenomenon. Correlations between the structures and fluorescence spectra of quenched chromophores have rarely been described.[10] Quenching efficiency is known to be proportional to $e^{-\beta d}$ (d = distance between the HOMO electrons of the quencher and the chromophore to be quenched). If the piperazine rings of **3** assume a chair conformation in which the methylene groups are in equatorial positions, the aniline nitrogen atoms appear to be too far apart from the xanthene ring (Scheme 2, $\mathbf{3}_{eq-eq}$) to effectively quench fluorescence through space by the PET mechanism.

Scheme 2. Conformational Analysis of Compound **3** by NMR.



To elucidate how these apparently too-distant aniline nitrogen atoms participate in PET quenching, we acquired COSY/NOESY/ROESY NMR data for compound **3** in DMSO-d_6 (Supporting Information).[11] NOE enhancement was observed between the anisole ring and the benzoic acid (Scheme 2, $\mathbf{3}_{ax-ax}$). These NMR experiments indicate that the flexibility of **3** is such that the *N*-(*p*-methoxyphenyl)-piperazine moieties are able to approach the xanthene ring. This proximity of the aniline nitrogen atoms to the xanthene ring of **3** explains why the *N*-(*p*-methoxyphenyl)piperazine moieties effectively quench the fluorescence of the xanthene ring through space.

To understand the role of *N*-arylpiperazine as a quencher of a xanthene system, a good control would be a DCF derivative with one *N*-arylpiperazine unit. However, the Mannich reaction of DCF, even when only 1 equiv of 1-(4-methoxyphenyl)piperazine was used, generated predominantly dialkylated DCF derivative **3**. Therefore, we have

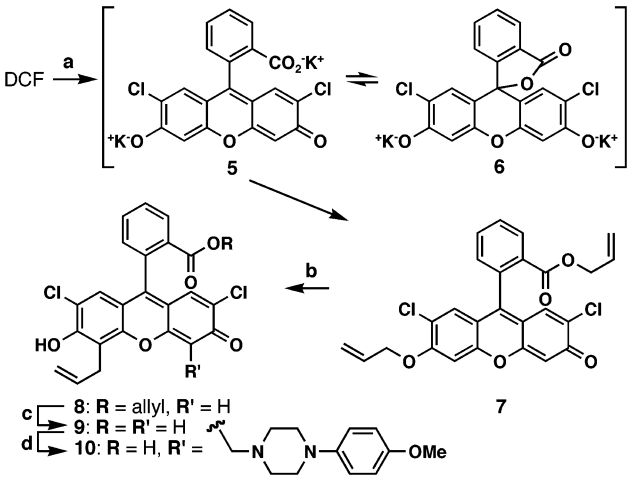
(9) Quantitative analysis of the correlation between the HOMO level of a quenching unit and the quantum yield is underway in our laboratory.

(10) Quenching of fluoresceinamine through covalent bonds was systematically studied previously: Munkholm, C.; Parkinson, D.-R.; Walt, D. R. *J. Am. Chem. Soc.* **1990**, *112*, 2608−2612.

(11) Compound **3** was not sufficiently soluble in D_2O for two-dimensional NMR studies. The quantum yield relationships in DMSO show the same trends as in pH 7 phosphate buffer.

developed a synthetic scheme to differentiate the 4′- and 5′-positions of DCF via the aromatic Claisen rearrangement. We first O-allylated DCF with allyl bromide to form compound **7** in 77% yield (Scheme 3). It is noteworthy that

Scheme 3. Synthesis of **10**[a]



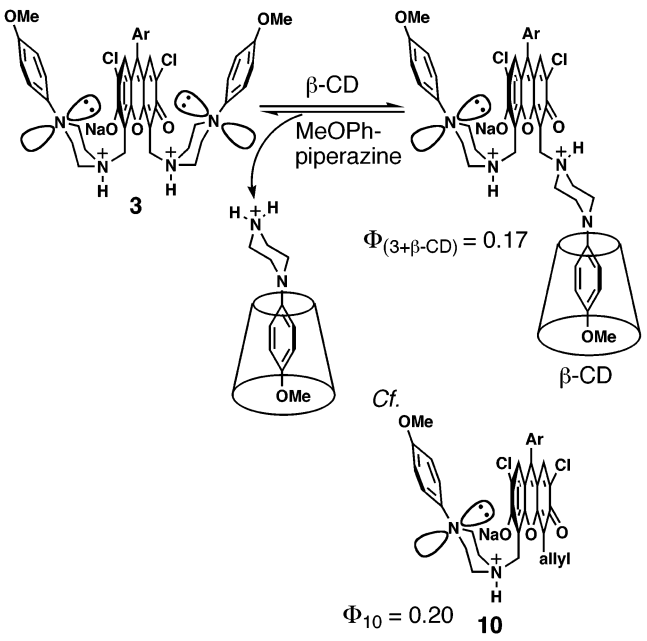
[a] Reagents and conditions: (a) allyl bromide (3.0 equiv), K_2CO_3 (3.0 equiv), DMF, 23 °C, 2.3 h, 77%; (b) Ph_2O, 150 °C, 1.5 h, 64%; (c) LiOH (5.0 equiv), 1,4-dioxane, H_2O, reflux, 1.3 h, 82%; (d) paraformaldehyde (10 equiv), 1-(4-methoxyphenyl)piperazine hydrochloride (3.0 equiv), $NaHCO_3$ (3.0 equiv), MeCN, reflux, 3.3 h, 65%.

we obtained the ester-ether allylation product, and no bis-aryl ether was formed. This unsymmetrical O-allylation set the stage for selective C−C bond formation. Upon heating of **7**, the aromatic Claisen rearrangement proceeded smoothly to afford the C-allylated DCF derivative **8** in 64% yield. The hydrolysis of ester **8** generated carboxylic acid **9** in 82% yield. Finally, the Mannich reaction using paraformaldehyde and 1-(4-methoxyphenyl)piperazine produced the desired compound **10** in 65% yield. The quantum yield of **10** was determined to be 20% of DCF (Table 1).

To test the reversibility of the quenching switch installed in compound **3**, we used β-cyclodextrin (β-CD)[12] to bind the anisole groups (Scheme 4), preventing the N-lone pair electrons from quenching the xanthene in the excited state. By titrating β-CD (final concentration: 5 mM) into a 5 μM solution of **3** in pH 7.4 phosphate buffer, the fluorescence intensity increased up to 4.6-fold.[13] The resulting fluorescence intensity (17% of DCF) is approximately the same as that of compound **10** (20% of DCF). The correlation of these intensities and the steric bulk of the β-CD leads us to speculate that a 1:1 complex is formed between **3** and β-CD. This correlation between free **10** and the **3**:β-CD complex suggests that the concentration-dependent unquenching of compound **3** occurred because β-CD bound to one of the two *p*-methoxyphenyl groups of **3**, thereby preventing the aniline nitrogen atoms from effectively interacting with the

(12) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.* **1998**, *98*, 1875−1917.
(13) We could not increase [β-CD] further due to its low solubility.

Scheme 4. Demonstration of a Reversible Quenching−Unquenching Switch System.



xanthene ring.[14] To verify that the β-CD was specifically binding to the switching moiety, 1-(4-methoxyphenyl)-piperazine was added as an antagonist to the mixture of **3** and β-CD, resulting in a 38% decrease in the fluorescence intensity (Supporting Information). This result can be explained by the competitive binding between **3** and 1-(4-methoxyphenyl)piperazine toward β-CD, further supporting our hypothesis (Scheme 4).

In summary, we have developed a general approach for designing and preparing reversibly quenched DCF derivatives. The quenching efficiency of the aniline nitrogen atoms can be tuned by changing the electron density of the covalently linked aromatic ring and by cooperative quenching. Moreover, our NMR and β-CD titration experiments correlate the fluorescence intensity to the conformations of DCF derivatives. We propose that binding of β-CD to the quenching side chain inhibits the quenching event through a conformational control mechanism. A new synthetic approach for desymmetrizing DCF enabled us to prepare the control compound **10**. The O-allylation−Claisen rearrangement sequence should find many applications in the preparation of unsymmetrical DCF derivatives.

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Supporting Information Available: Synthetic procedures and spectroscopic data for **1**−**4** and **7**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) We are currently investigating this relationship in more detail.