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One-Pot Synthesis of Tetrafluoro- and Tetrachlorofluorescein Derivatives and Their Stabilization by β-Cyclodextrin

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A highly efficient one-pot procedure for the preparation of fluorescein-based dyes with relatively high yield was investigated. Significantly, introduction of these fluoro and chloro functional groups and forming host-guest complexes with cyclodextrins partially enhanced the photostability of these dyes.

Keywords fluorescein, cyclodextrin, photostability, cucurbit[7]uril, one-pot synthesis, supramolecular chemistry

Introduction

Since the first synthesis in 1871 by von Bayer,[1] fluoresceins in dianion form, due to their brightness, excellent fluorescence quantum yields, low-energy excitation and emission wavelengths, and good biocompatibility, have shown wide-spread applications in many research fields, such as single molecule detection, fluorescence labeling, dye lasers, conversion and storage of solar energy, fluorescence-based assays, and cell staining and antitumor agents.[2] However, facile synthesis of new analogues of fluoresceins is still in urgent need in order to further improve their properties and overcome the disadvantages of current dyes, such as the instability of fluorescein conjugates, irreversible photo-bleaching by a powerful laser, and strong pH dependence of fluorescence in aqueous solutions.[3]

Cyclodextrins (CDs) and cucurbit[*n*]urils (CB[*n*]s) are two kinds of functional macrocyclic compounds, which can selectively form inclusion complexes with organic guests.[4] Recently, Nau *et al.*[2a] reported that rhodamine 6G could be made ultrastable upon complexation with CB[7]. Herein, we wish to describe a new method to obtain photostable fluoresceins. We not only discussed the contribution of functional groups to the stability of the dyes in dianion form, but also explored the influence of the formation of inclusion complexes upon binding with CDs or CB[*n*]s.

Experimental

Unless otherwise noted, commercial reagents were used as received and all reactions were carried out under ambient condition. Fluorescein, 4,5,6,7-tetrafluoroisobenzofuran-1,3-dione, 4,5,6,7-tetrachloroisobenzofuran-1,3-dione, β-CD were reagent grade and purchased from Aladdin Co. Ltd. Deionized water was used in all experiments. ^{1}H NMR spectra were collected at 25 ℃ on a Varian-300 MHz NMR spectrometer. ^{13}C NMR spectra were recorded at 25 ℃ on a Varian 75 MHz NMR spectrometer and Varian 151 MHz NMR spectrometer. ^{19}F NMR spectra were recorded at 25 ℃ on a Varian 565 MHz NMR spectrometer. Mass spectra were recorded on Liquid ChroMatography. Mass Spectrometry Instrument (Agilent1290-micrOTOF Q II). UV-vis spectra were recorded on a Shimadzu UV-2550 instrument and all fluoresceins are in dianion form. The pK_{a2} of fluorescein is 6.5, the pK_{a2} of compound **1** is 6.1, and the pK_{a2} of compound **2** is 5.97, when β-CD was added the pK_{a2} of these dyes changed to 6.35, 6.67 and 7.40 (see the supporting information).[5] The NMR titration experiments were performed in basic condition with 2 mmol/L of K_2CO_3 in D_2O, which pH is measured to be 10.82.

4,5,6,7-Tetrafluorofluorescein (1)

In a 100 mL round-bottom flask, 4,5,6,7-tetrafluoroisobenzofuran-1,3-dione (4.76 g, 20 mmol) was dissolved in $MeSO_3H$ (21 mL) at 80 ℃ before concentrated H_2SO_4 (6 mL) was added. The reaction mixture was heated at 90 ℃ under vigorous stirring after resorcinol (5.51 g, 50 mmol) was added in three portions. After 2 h, the reaction mixture was heated up to 120 ℃ under vigorous stirring, and was allowed to react for another 4 h. The reaction mixture was cooled down to room temperature and quenched with H_2O (100 mL). The solid precipitate was suction filtered and washed with H_2O, then dried *in vacuum* to give **1** (6.65 g, yield: 92%). ^{1}H NMR (300 MHz, CD_3SOCD_3, 25 ℃) δ: 10.27 (s, 2H), 7.01 (d, J=8.7 Hz, 2H), 6.70 (d, J=2.0

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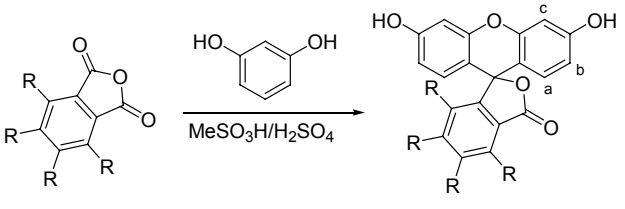
Hz, 2H), 6.60 (dd, J=8.7, 2.2 Hz, 2H); ^{13}C NMR (151 MHz, CD_3SOCD_3, 25 ℃) δ: 170.38, 162.58, 160.37, 160.29, 156.30, 155.65, 152.18, 151.82, 151.33, 145.97, 145.86, 145.76, 144.56, 144.48, 144.23, 144.13, 144.03, 142.82, 142.73, 142.40, 141.58, 140.81, 140.72, 139.96, 133.10, 133.02, 128.99, 128.78, 126.71, 126.14, 122.42, 113.15, 112.90, 111.40, 111.32, 107.26, 107.00, 103.62, 102.50, 102.31, 81.68, 80.75, 59.79, 20.77, 14.10; ^{19}F NMR (565 MHz, CD_3SOCD_3, 25 ℃) δ: −140.12 (d, J=15.8 Hz, 1F), −144.04 (t, J=15.7 Hz, 1F), −144.21 (t, J=19.2 Hz, 1F), −151.97 (t, J=20.1 Hz, 1F). m.p.＞300 ℃. ESI-HRMS calcd for $C_{20}H_9F_4O_5$ 405.0381, found 405.0395.

4,5,6,7-Tetrachlorofluorescein (2) 4,5,6,7-Tetrachloroisobenzofuran-1,3-dione (0.2859 g, 1 mmol) was dissolved in $MeSO_3H$ (1.25 mL) at 90 ℃, and resorcinol (0.2753 g, 2.5 mmol) was added in three portions. After 2 h of reaction, the mixture was stirred at 125 ℃ for 4 h, and then cooled down to room temperature and quenched with H_2O (12.5 mL). The solid precipitate was suction filtered and washed with water, then dried *in vacuum* to give **2** (0.2928 g, yield 82%). ^{1}H NMR (300 MHz, CD_3SOCD_3, 25 ℃) δ: 10.19 (s, 2H), 6.94 (d, J=8.7 Hz, 2H), 6.67 (d, J=2.3 Hz, 2H), 6.55 (dd, J=8.7, 2.4 Hz, 2H); ^{13}C NMR (75 MHz, CD_3SOCD_3, 25 ℃) δ: 163.5, 159.9, 148.3, 134.8, 128.9, 124.1, 112.6, 106.6, 102.3, 81.6. m.p.＞300 ℃. ESI-HRMS calcd for $C_{20}H_9Cl_4O_5$ 468.9199, found 468.9216.

Results and Discussion

Fluorescein and its derivatives were synthesized by condensation of appropriate resorcinol with various derivatives of phthalic anhydride in the presence of various acid catalysts, *e.g.*, $MeSO_3H$,[5] AcOH-HCl,[6] CsF[7] and $ZnCl_2$.[1] But these conventional methodologies for the synthesis of fluorescein and derivatives suffer from relatively high cost, low yield, hazard materials, high temperature and long process. Herein, a novel methodology for preparing dyes and switchable dyes is shown in Scheme 1. It is a technological break-through that enables us to prepare conventional or special functional dyes in a simple one-pot, low cost and highly efficient process. $MeSO_3H$ served as both a suitable solvent and a Lewis acid catalyst in the dye-forming reaction. On the other hand, H_2SO_4 was introduced to increase the acidity strength, and gradually increase of reaction temperature effectively avoided the sublimation. The yields of the obtained fluorescein derivatives (**1** and **2**) were highly improved and the whole synthetic protocol and purification progress were very straightforward.

Scheme 1 One-pot synthetic route to the fluorescent dyes (**1** and **2**) with $MeSO_3H$-H_2SO_4 as catalyst



1 R = F
2 R = Cl

Although fluorescein and its derivatives share a common, xanthene-based skeleton, different substituents can be made to cause marked differences in absorbance and fluorescence emission wavelengths. Selective substitution of aromatic hydrogen of fluoresceins with chlorine has been seen to increase fluorescence efficiency and to narrow emission and absorbance maxima.[8] The absorption peak of the chlorinated fluorescein (**2**) was found to be red-shifted by 25 nm (Figure 1), which may be due to the electron-withdrawing ability of the chlorine substituted group.[9]

The substitution of hydrogen atoms by fluorine atoms in organic compounds often results in profound changes in their properties, largely due to the highly electro-negative nature and small van der Waals radius of the fluorine atom.[10] Unlike other halogenated compounds, in which an expected pattern derived from the substitution effect could be inferred, fluorination of organic compounds often results in products with unexpected properties. These halogenated derivatives undergo significant intersystem crossing to the triplet state after light absorption.[11] In our research for the fluorination of fluorescein (**1**) to improve its properties, we sought to retain the favorable characteristics of fluorescein, especially high absorbance and absorption at long wavelength, which were very useful as molecular probes. Fluorination of fluorescein permits us to examine the influence of fluorine on this widely used fluorescent molecule.[5a]

Photo-bleaching is a dynamic process by which a fluorophore undergoes a photo-induced chemical destruction upon exposure to light and thus loses its ability to fluoresce.[12] The photo-bleaching mechanism for a fluorophore present in biological systems is a complicated process on which many studies have been focused. The photo-bleaching behavior of free and bound fluorescein has also been investigated by experimental methods. Both the theoretical simulation and experimental data show that photo-bleaching of fluorescein involved three steps of reactions that could lead to irreversible bleaching photoproducts,[5a] (a) the reactions of two triplet dyes to form semireduced (R) and semioxidized (X) form of dyes; (b) the reaction of triplet dye with ground-state dye to form R and X; (c) 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 for the introduction of these two kinds of groups indicates that at least one of the three reactions is affected assuming other conditions remain the same (Figure 1). There are two possible ex-

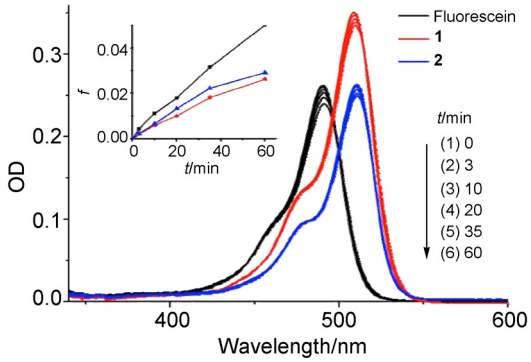


Figure 1 Photo-bleaching of fluorescein (4 μmol/L), **1** (4 μmol/L), and **2** (4 μmol/L) in a phosphate buffer solution (pH 8.0) in the absence of *β*-CD and CB[7] followed through the increasing time of a 250-W mercury arc lamp irradiation. The inset shows the graph and correlation lines for the characteristic function $f=\log([10^{A0}-1]/[10^{A}-1])$ versus irradiation time for the determination of the relative quantum yield of photo-bleaching.[13]

planations that account for the improved resistance to photo-bleaching: (a) the introduction of group could shorten the triplet lifetime, thus decreasing the probability of its reaction with a quencher; (b) the incorporation of these groups 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. And, the degree of the stabilization depends on electro-withdrawing ability of these groups, *i.e.*, the higher of the electro-withdrawing ability of the substituents, the more photostable of the fluorescein derivatives.

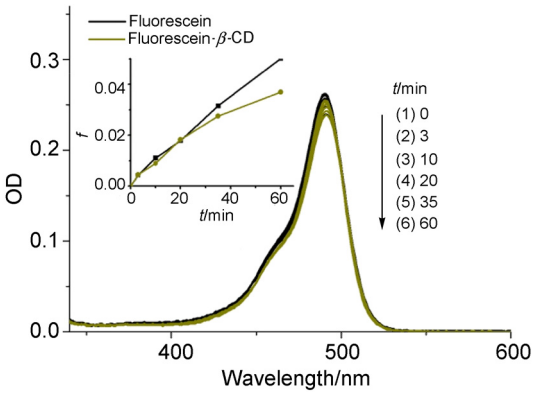


Figure 2 Photo-bleaching of fluorescein (4 μmol/L) in phosphate buffer (pH 8.0) in the absence and presence of *β*-CD (2 mmol/L) with an increasing time of irradiation by a 250 W mercury arc lamp.

Supramolecular complexation of chromophoric guest molecules by macrocyclic hosts can affect their fluorescent properties as a consequence of an altered microenviroment.[14] As reported, the addition of CB[7] to aqueous solutions of some important cationic fluorescent dyes has exposed a synergistic interplay of several advantageous effects, which include increased quantum yields, increased photostability, high brightness, spectral shifts, prevention of unspecific adsorption and dye aggregation.[2a] While anionic centers result in low binding,[15] expectedly, the anionic dye fluorescein does not form a complex with CB[7]. We introduced CB[7] into the solutions of fluorescein and its derivatives (**1** and **2**), the administration of CB[7] does not have any influence on improving the photo-bleaching of the synthesized dyes as expected (see the supporting information). Meanwhile, due to the solvation effect caused by a higher concentration of CB[7], the absorption strength was reduced.

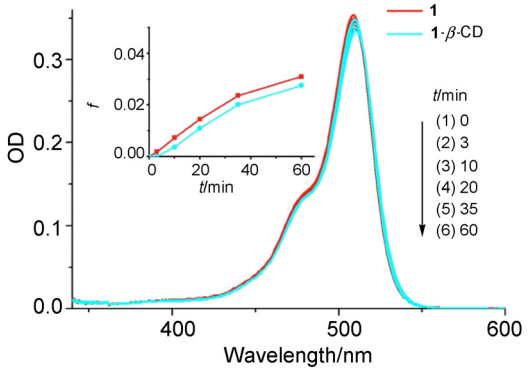


Figure 3 Photo-bleaching of **1** (4 μmol/l) in phosphate buffer (pH 8.0) in the absence and presence of *β*-CD (2 mmol/L) with an increasing time of irradiation by a 250 W mercury arc lamp.

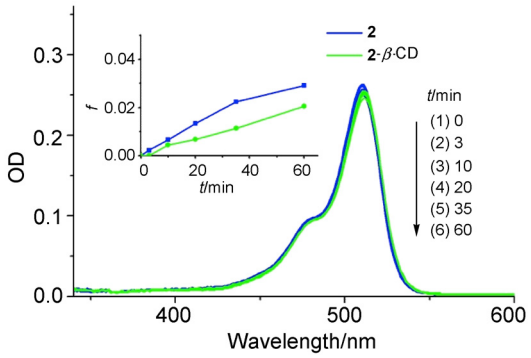


Figure 4 Photo-bleaching of **2** (4 μmol/L) in phosphate buffer (pH 8.0) in the absence and presence of *β*-CD (2 mmol/L) with an increasing time of irradiation by a 250 W mercury arc lamp.

β-CD and its various derivatives are well known, possessing a truncated cone with hydrophilic outer surface and hydrophobic internal cavity.[5c] Flamigni[16] and Buvári *et al.*[17] reported the association constants of *β*-CD and fluorescein at basic pH and acidic pH to be (360±40) L•mol^{-1} and (1100±100) L•mol^{-1}, respectively. As is apparent from Figures 2—4, there is an increase in the photostability upon addition of *β*-CD (With longer time absorbing, photoproducts are formed, which cause the curve to become nonlinear), and there is no difference when we increase the concentration of *β*-CD to 18 mmol/L (see the supporting information). Apparently, *β*-CD selectively prevents the two-step

two-photon photolysis, and does not show any common drawbacks. In another words, it does not act as a quencher, does not absorb in the visible region, and consequently displays non-fluorescence. These combined properties distinguish *β*-CD from other stabilizing additives.[18]

The photostablizing effect of *β*-CD is unquestionably related to a combination of factors, involving the inert cavity, its low polarizability, the confinement of the dye, and effective protection from water and oxygen. As reported, glucose and alcohols were used to confirm the formation of supramolecular complexes, and that the improvement of the photostabilities is not caused by the solvent effect but by a higher concentration of *β*-CD.[19] Analysis on the chemical shifts of the ^{1}H NMR signals of the complex in relation to the signals from the pure *β*-CD and **2** in dianion state with excess K_2CO_3, are the main indication of the extent of the complex formation (Figure 5). The association constants of β-CD with **1** and **2** were determined to be (122±15) L•mol^{-1} and (637±70) L•mol^{-1} (Figure 6), respectively, which ensures the complete complexation between dyes and *β*-CD in our photo-bleaching experiments (see the Supporting Information).

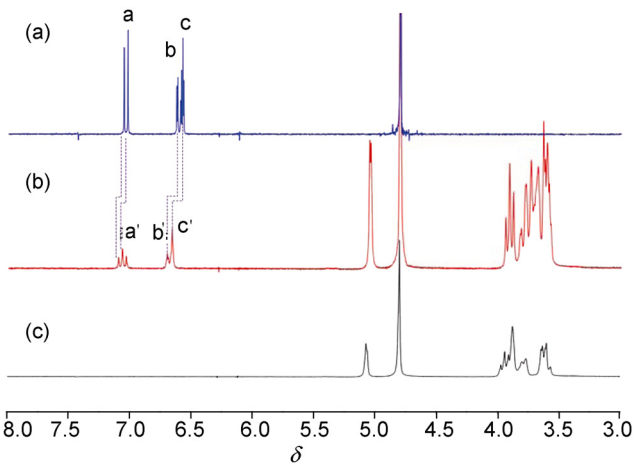


Figure 5 ^{1}H NMR spectra in D_2O with excess K_2CO_3 (pH 10.82), the molar ratio of **2** and *β*-CD was 1∶1 to get the complex: (a) 10 mmol/L **2**; (b) 10 mmol/L **2** and 10 mmol/L *β*-CD; (c) 10 mmol/L *β*-CD.

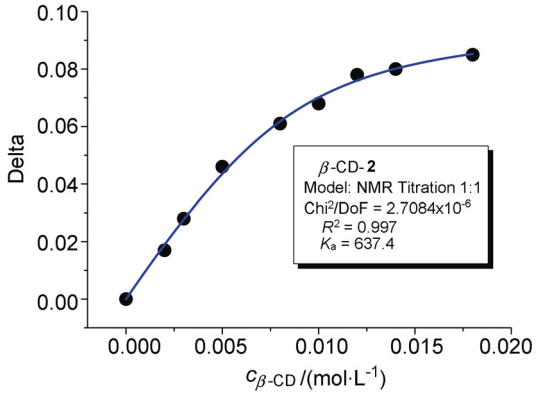


Figure 6 The non-linear curve-fitting (NMR titrations) for the complexation of *β*-CD (8.4 mmol/L) with **2** in D_2O at 298 K. The concentration of **2** was 0, 2, 3, 5, 8, 10, 12, 14, and 18 mmol/L, respectively.

β-CD has a stronger effect on the photostabilization of **2**, due to the binding interaction of *β*-CD and this fluorescein derivative within the cavity. The structure of guest is very important for host-guest inclusion complexation. The shape and size of the guest must match the cavity of *β*-CD, and its polarity should be smaller than water. As compared with fluorescein and **1**, the hydrophobicity of **2** is relatively stronger, which made it easier to enter the hydrophobic cavity of *β*-CD forming a relatively stable inclusion complex.

Effort has been made to improve the photostabilizing properties of organic dyes,[20] numerous additives have been tested to date but showed in common limited photostabilizing properties on organic dyes, *i.e.*, incompatibility with biologically or environmentally relevant applications, use in large scale, operable either by preventing secondary photolytic steps or by quenching undesired reactive triplet states, *etc*. However, the addition of *β*-CD provides an alternative, supramolecular approach to achieve increased photostabilization of fluorescein-based dyes, as reported in this work. Significantly, *β*-CD is transparent in the visible wavelength range and does not act as fluorescence quencher for dyes at a range of concentrations.

Conclusions

We have developed a new method for the synthesis of fluorescein derivatives in high yields. Dye **1** has better photophysical properties, *i.e.*, high brightness, good solubility in water, increased photostability, long absorption wavelength as compared with fluorescein and **1**. The addition of *β*-CD to form supramolecular inclusion complex increased the photostabilities of fluorescein and its derivatives, showing potential applications of CDs in organic dye industry.

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