Intramolecular Fluorescence Resonance-energy-transfer of Fluorescein Derivative; Phenyl and Styryl p-Disubstituted Fluorescein

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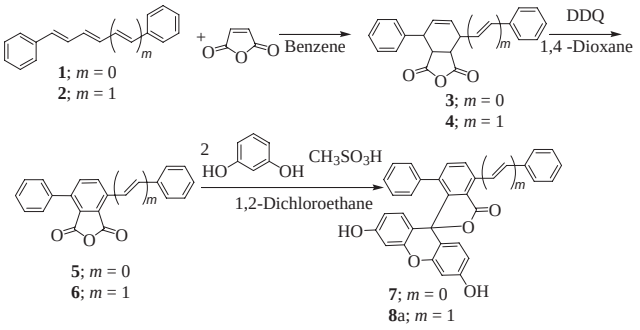
We describe synthesis and fluorescence properties of phenyl and styryl p-disubstituted fluorescein. The compound was synthesized in three reaction steps from *trans*,*trans*,*trans*-1,6-diphenyl-1,3,5-hexatriene. The fluorescence spectrum of the product in alkaline methanol solution suggested occurrence of intramolecular FRET from the donor site of the benzene moiety to the acceptor site of the xanthene moiety.

In recent years, considerable effort has been devoted to developing new fluorescence dye-based probes as excellent sensors for biomolecules, being sensitive, fast-responding, and capable of affording high spatial resolution via microscopic imaging.[1] Among many fluorescent dyes, fluorescein is known to have a high quantum yield of fluorescence in aqueous solution and to be excitable at long wavelength.[2] The fluorescein is appropriately divided into two parts, i.e., the benzene moiety and the xanthene moiety.[3] The fluorescein can be prepared by the condensation reaction of phthalic anhydride with resorcinol under acidic conditions.[4] However, little has been reported to prepare fluorescein derivatives directly having p-substituents with π conjugation on the benzene moiety.

The fluorescence intensity of the probes is influenced by many factors, such as the changes of environment around the probe (pH, polarity, and temperature) and the changes in the concentration and the excitation intensity. To reduce the influence of the factors, ratiometric measurements are utilized,[5] in which the fluorescence intensities are simultaneously recorded and their ratio is calculated. This allows more precise measurement than that at a single wavelength. To perform the ratiometric measurement, the probes necessarily exhibit a large shift in their emission or excitation spectrum after they react or bind with the target molecules. A fluorescence resonance-energy-transfer (FRET)[6] technique has been used in the designed fluorescent probes to obtain a large shift in the spectroscopic peak. FRET is an interaction between the electronic excited states of two fluorophores, in which excitation energy is transferred from a donor to an acceptor without emission of a photon.[7] In previous studies, intramolecular FRET compounds bearing a fluorescein acceptor and a dye-based donor such as coumarin have been synthesized.[8] In the compounds, the donors and acceptors are connected by flexible linkers such as ethylene glycols and phosphates, and the excitation energy of the coumarin donor is transferred to the fluorescein acceptor.

Recently, we reported the synthesis of poly(*p*-phenylene) having fluorescein moieties in the main chain and the analogous compound **7** of its repeating unit structure.[9] As shown in Scheme 1 for the synthesis of **7**, Diels–Alder reaction of *trans*,*trans*-1,4-diphenyl-1,3-butadiene (**1**) with maleic anhydride, followed by aromatization with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave 3,6-diphenylphthalic anhydride (**5**).[10]



Scheme 1. Synthesis of diphenyl p-disubstituted fluorescein **7** and phenyl and styryl p-disubstituted fluorescein **8a** (**8b** is a regioisomer of **8a**).

Then, the reaction of **5** with resorcinol was conducted in the presence of methanesulfonic acid in 1,2-dichloroethane, giving rise to diphenyl p-disubstituted fluorescein **7**. Both the polymer and the analogous compound **7** have the π-conjugated structures at the benzene moieties, and accordingly, the above synthetic route can be considered to be useful for the preparation of various fluorescein derivatives having π-conjugated structures at the benzene moieties.

On the basis of the above viewpoints, in this letter, we report the synthesis of a new fluorescein derivative, i.e., phenyl and styryl p-disubstituted fluorescein **8** according to the synthetic route of Scheme 1, which has longer π conjugation at the benzene moiety than that of **7**. Furthermore, we found interesting fluorescence properties of **8**, in which intramolecular FRET from the benzene moiety to the xanthene moiety in the fluorescein structure occurred, which is possibly applicable to the ratiometric measurement.

As the first step, we performed Dields–Alder reaction of a starting compound, *trans*,*trans*,*trans*-1,6-diphenyl-1,3,5-hexatriene (**2**)[11] with maleic anhydride to give **4**.[10,12] The ^{1}H NMR ($CDCl_3$) and IR spectra of the isolated product fully supported the structure of **4**. Then, the aromatization of **4** was carried out using DDQ at 60 °C in 1,4-dioxane.[13] The ^{1}H NMR spectrum ($CDCl_3$) of the isolated product showed the disappearance of the signals due to the cyclohexene group in **4**, indicating the occurrence of perfect aromatization. The resulting **6** was converted into the desired compound **8** by the reaction with resorcinol in the presence of methanesulfonic acid in 1,2-dichloroethane at 40 °C.[3,4] After the reaction was performed for 375 h, the crude product was purified by column chromatography on silica gel to give a mixture of two regioisomers **8a** and **8b** (the structure of **8b** is shown in Figure S3[14]). The structures were confirmed by the ^{1}H NMR spectrum (Figure S3,[14] DMSO-d_6 containing a small amount of D_2O). The molar ratio of **8a** to **8b** was calculated by the integrated ratio of the NMR signals to be 85:15.

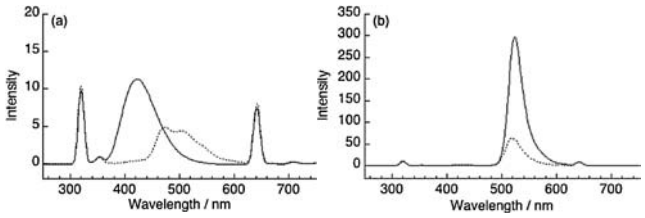


Figure 1. Fluorescence spectra of **8** (—) and fluorescein (- - -) (0.50 μmol/L); excited at 320 nm in 0.50 mmol/L HCl/methanol (a) and excited at 320 nm in 0.50 mmol/L NaOH/methanol (b).

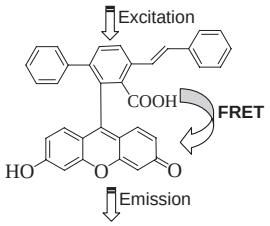


Figure 2. An image for intramolecular FRET from benzene moiety to the xanthene moiety in **8**.

The conversion of **6** into **8** was also supported by the IR spectrum, in which the carbonyl absorptions due to the carboxylic anhydride group disappeared, whereas the new carbonyl absorption ascribable to the lactone structure in the fluorescein appeared at 1735.8 cm^{-1}. The isolated yield of **8** was 8%, which was much lower than that of **7** formed from **5** by the same procedure. Although the lower yield in the reaction of **6** with resorcinol was probably caused by the production of a large amount of by-products, the reason for the low yield is not yet clear.

The fluorescence spectrum of **8** (Figure 1a) excited at 320 nm in 0.50 mmol/L HCl/methanol shows an emission maximum peak at 420 nm, which is probably ascribed to the π^*–π transition of the benzene moiety in **8**, whereas no emission peak is observed at the same wavelength region in the fluorescence spectrum of a standard fluorescein. Interestingly, when the fluorescence spectrum of **8** is measured with excitation at 320 nm in 0.50 mmol/L NaOH/methanol, a typical emission maximum at 520 nm, attributed to the quinoid form of fluorescein by ring opening under the alkaline conditions, is observed, but no emission due to π^*–π transition is seen (Figure 1b). These results indicate that FRET from the benzene moiety to the xanthene moiety in **8** occurs as shown in Figure 2. This FRET is possibly caused by the intramolecular overlap integral between the emission spectrum of the benzene donor site and the absorption spectrum of the xanthene acceptor site in **8**. Indeed, the broad emission peaks ascribable to π^*–π transition of **6** (as a standard compound for the structure of the phenyl and styryl p-disubstituted benzene moiety) excited at 320 nm in 0.50 mmol/L NaOH/methanol certainly overlapped with the absorptions at 420–520 nm of the standard fluorescein in the same alkaline conditions (Figure S4[14]).

The emission intensities at 520 nm in the fluorescein spectra of the standard fluorescein (Figure 1b) and **7** (data not shown) excited at 320 nm in 0.50 mmol/L NaOH/methanol were much lower than those of **8**, indicating that no obvious FRET happened between the benzene and xanthene moieties in these compounds. Generally, FRET takes place through the resonance interaction between the donor and acceptor rather than their strong electronic coupling interaction, which affects the absorption spectrum. The absorption spectral pattern of **8** in 0.50 mmol/L NaOH/methanol (Figure S5a[14]) is similar as the superimposed pattern of the absorption spectra of **6** and fluorescein in the same solvent (Figures S5b and 5c[14]), supporting no strong electronic interaction between the benzene and xanthene moieties in **8**.

In conclusion, we have synthesized phenyl and styryl p-disubstituted fluorescein **8** according to Scheme 1. The ^{1}H NMR spectrum of the isolated product indicated that it was a mixture of the regioisomers **8a** and **8b**. The fluorescence spectrum of **8** excited at 320 nm in 0.50 mmol/L NaOH/methanol indicated occurrence of the intramolecular FRET between the benzene and xanthene moieties. This fluorescence property has a possibility for developing a novel ratiometric fluorescent probe as a pH sensor.

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