

New Fluorescent Labels: 4- and 7-Chlorofluorescein

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A new mixture of 4- and 7-chlorofluorescein were synthesized by condensation of resorcinol with 3-chlorophthalic anhydride in the presence of methanesulfonic acid or zinc chloride. These regioisomers were successfully separated by chromatography. The photophysical properties were examined and their absorption and emission maxima at long wavelength, high fluorescence quantum yield, and narrow emission bandwidth were found, highly favorable for detecting multiple target substances in the same sample. Furthermore, 4(7)-chlorofluorescein were found to be strongly pH-dependent between 4.0 and 8.0, and could be used as pH-sensitive fluorescent probe to measure intracellular pH.

KEY WORDS: Fluorescent probe; 4(7)-chlorofluorescein; synthesis; fluorescent properties.

INTRODUCTION

Fluorescein is the most commonly used labels because of its solubility in aqueous buffers and high fluorescence quantum yield [1,2]. Over the years, a wide variety of fluorescein derivatives have been prepared and used as fluorescent detection reagents [3–8]. In general, the selective substitution of chlorine for aromatic hydrogen in organic compounds results in profound changes in their photophysical properties [9]. Therefore, it is very important to study the fluorescein derivatives substituted by chlorine. However, the preparation and photophysical properties of 4- and 7-chlorofluorescein have not been reported previously.

In this paper, 4(7)-chlorofluorescein was synthesized and separated by chromatography with the eluent of 1:20 concentrated ammonia/ethanol. And the pure regioisomers **1a**(7-chlorofluorescein) and **1b**(4-chlorofluorescein) (as Fig. 1) were characterized by NMR, MS, and fluorescent spectroscopy.

EXPERIMENTS

4- and 7-Chlorofluorescein Preparation

The synthesis and separation of 4(7)-chlorofluorescein are outlined in Fig. 2. Two routes A and B for the preparation of the mixture 4(7)-chlorofluorescein are investigated in this paper.

Route A: Resorcinol (5.50 g, 0.050 mol) and 3-chlorophthalic anhydride (3.67 g, 0.020 mol) were crushed and melted at 130°C. Fused ZnCl₂ (7.09 g, 0.052 mol) was added slowly and the temperature was slowly increased to 150–160°C over 2 hr until the material solidified. The brick red solid was pulverized and boiled in 80 mL of 1 M HCl for 1 hr. The red solid was collected on a frit, washed with hot water, and dried in vacuo. Crude product (7.1 g) was obtained in 76% yield. The mixture of 4(7)-chlorofluorescein were separated by column chromatography on silica with the eluent of 1:20 concentrated ammonia/ethanol.

Route B: 3-chlorophthalic anhydride (3.67 g, 0.020 mol) was added to a solution of resorcinol (5.50 g, 0.050 mol) in 40 mL methanesulfonic acid. The resulting suspension was heated under N₂ at 85°C for 8 hr. The cooled mixture was poured into 7 volumes of ice water, followed by filtration. The filtrate was dried and gave crude product (8.5 g, yield 91%). The mixture of 4(7)-chlorofluorescein were separated by column

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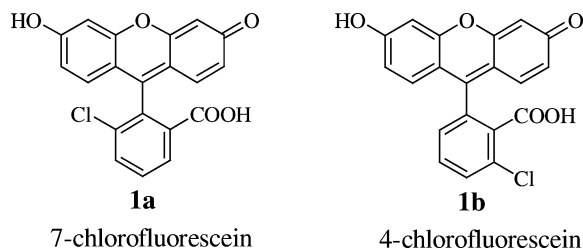


Fig. 1. The structure of 4- and 7-chlorofluorescein.

chromatography on silica with the eluent of 1:20 concentrated ammonia/ethanol.

Compound **1a** (7-chlorofluorescein): mp 242–243°C; $^1\text{H NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ 10.12 (2H, s), 7.79 (1H, *d*, $J = 7.4$ Hz), 7.81 (1H, *d*, $J = 7.2$ Hz), 7.73 (1H, *t*, $J = 7.8$ Hz), 6.67 (2H, *d*, $J = 2.2$ Hz), 6.62 (2H, *d*, $J = 8.7$ Hz), 6.50 (2H, *dd*, $J = 2.6, 8.9$ Hz); $^{13}\text{C NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ 167.31, 159.89, 152.63, 147.96, 135.73, 133.56, 132.21, 128.73, 128.38, 127.91, 111.43, 105.94, 102.23; MS(ESI-TOF): found 367.3(M+H)⁺, calcd 366.8.

Compound **1b** (4-chlorofluorescein): mp 244–246°C; $^1\text{H NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ 10.12 (2H, s), 7.80 (1H, *d*, $J = 7.2$ Hz), 7.82 (1H, *d*, $J = 7.6$ Hz), 7.74 (1H, *t*, $J = 7.6$ Hz), 6.66 (2H, *d*, $J = 2$ Hz), 6.63 (2H, *d*, $J = 8.4$ Hz), 6.54 (2H, *dd*, $J = 2.4, 8.8$ Hz); $^{13}\text{C NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ 167.51, 159.61, 151.88, 147.90, 135.93, 132.44, 128.73, 128.32, 128.28, 123.91, 112.58, 107.44, 102.25; MS(ESI-TOF): found 367.3 (M+H)⁺, calcd 366.8.

Photophysical Properties

UV–Visible Spectroscopy

Absorption spectra were recorded on a UV-2450 (Shimadzu). ϵ was denoted molar extinction coefficient. It was calculated by the following Eq. (1).

$$\epsilon = \frac{A}{bc} \quad (1)$$

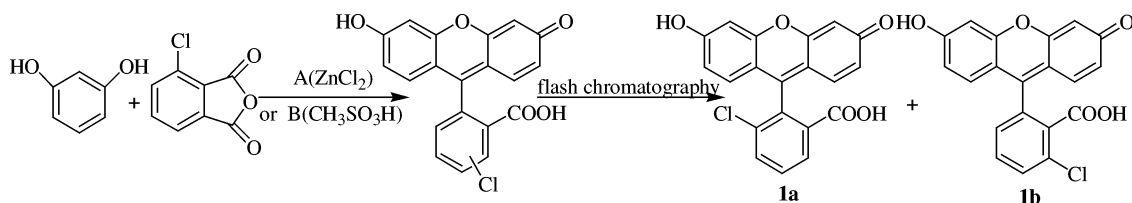


Fig. 2. The synthesis of **1a** (7-chlorofluorescein) and **1b** (4-chlorofluorescein).

Fluorescence Spectroscopy

Fluorescence spectra were recorded on JASCO FP-750 spectrofluorimeter. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by rhodamine correction curve. Spectra were routinely acquired at 25°C, maintained by a circulating water bath. All emission spectra of these compounds were detected by the same instrument and the same concentration.

Quantum Yield

ϕ denoted the quantum yield for fluorescence. It was obtained by comparison of the integrated area of the corrected emission spectrum of the sample with that of a solution of fluorescein in 0.1N NaOH, which has a quantum efficiency of 0.9 [10]. ϕ can be calculated from multiple measurements ($N = 3$) with the following Eq. (2) (Abs was obtained from the absorption spectra and $\int F$ was calculated by summation of fluorescence intensity).

$$\phi_{\text{sample}} = \frac{Abs_{\text{standard}} \phi_{\text{standard}} \int F_{\text{sample}}}{Abs_{\text{sample}} \int F_{\text{standard}}} \quad (2)$$

pH-Dependent Fluorescence Studies

The fluorescence emission of 4(7)-chlorofluorescein at 499 nm were measured for pH variations ranging from 2 to 10.

DISCUSSION

Syntheses of 4- and 7-Chlorofluorescein

According to Route A, the devised products were obtained by fusion of resorcinol, 3-chlorophthalic anhydride, and zinc chloride in 76% yield. Because of the higher reaction temperature, considerable amounts of the starting materials were lost due to sublimation, which ac-

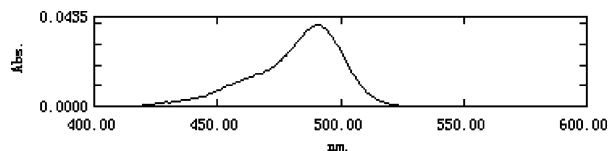


Fig. 3. UV-Vis spectra of fluorescein.

counted for the slight lower yield. According to Route B, the mixture of 4(7)-chlorofluorescein were synthesized by condensation of resorcinol with 3-chlorophthalic anhydride in methanesulfonic acid at 85°C in 98% yield. The methanesulfonic acid served as both a suitable solvent and a Lewis acid catalyst in this reaction. Route B is an improved procedure with high yield under mild condition. Despite the ease of preparation of the mixture 4(7)-chlorofluorescein, it is difficult to obtain pure regioisomer **1a** and **1b**. Attempts to generate **1a** and **1b** by the literature procedure [11–12] for separation of 5(6)-halogenated fluoresceins and 5(6)-carboxylfluorescein by fractional crystallization did not work properly in this case. However, we found that the mixture of 4- and 7-chlorofluorescein could be separated by column chromatography on silica with the eluent of 1:20 concentrated ammonia/ethanol. The pure regioisomers were confirmed by MS, ¹H NMR, and ¹³C NMR.

Fluorescent Properties

The fluorescent properties of 4(7)-chlorofluorescein were investigated by comparison with fluorescein and 2',7'-dichlorofluorescein [¹H NMR (400 MHz, DMSO-*d*₆) δ 11.08 (2H, s), 8.02 (1H, *d*, *J* = 10.0 Hz), 7.86–7.73 (2H, m), 7.34 (1H, *d*, *J* = 10.4 Hz), 6.91 (2H, s), 6.66 (2H, s); ¹³C NMR (400 MHz, DMSO-*d*₆) δ 168.97, 155.82, 152.19, 150.75, 136.62, 131.22, 128.90, 126.58, 125.78,

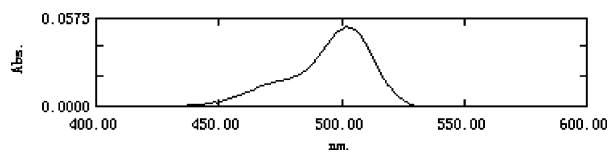


Fig. 4. UV-Vis spectra of 2',7'-dichlorofluorescein.

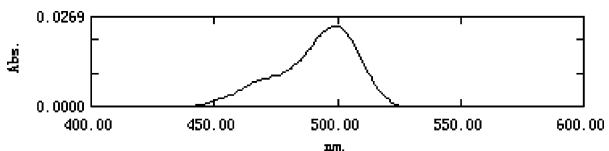


Fig. 5. UV-Vis spectra of 4(7)-chlorofluorescein.

124.65, 116.93, 111.13, 104.38, 82.16; MS(ESI-TOF): found 401.2 (M+H)⁺, calcd 400.0]. As can be seen from the UV-Vis spectra (Figs. 3–5), 2',7'-dichlorofluorescein and 4(7)-chlorofluorescein have been found to shift the spectra toward longer wavelengths. From Fig. 6, the fluorescence excitation and emission spectra of 4(7)-chlorofluorescein, it had excitation maxima (λ_{ex}) at 499 nm, emission maxima (λ_{em}) at 521 nm. The wavelength of absorption maxima (λ_{ab}), molar extinction coefficient (ϵ), the wavelength of fluorescence emission maxima (λ_{em}), Stokes shift, emission band halfwidth, and fluorescence quantum yield (ϕ) are summarized in Table I for three compounds. The 4(7)-chlorofluorescein had absorption maxima (λ_{ab}) at 499 nm, emission maxima (λ_{em}) at 521 nm. Comparison with fluorescein, λ_{ab} and λ_{em} produced a red shift with the electron-withdrawing ability of chlorine substituted group. And its fluorescence quantum yield is higher than fluorescein, the reason may be that 4(7)-chlorofluorescein, which is substituted by chlorine, have a lower population of triplet relative to singlet. 2',7'-dichlorofluorescein has similar fluorescent properties as 4(7)-chlorofluorescein with chlorine substituted group. As shown in Fig. 7, the emission bandwidth of 4(7)-chlorofluorescein is narrower than either of fluorescein and 2',7'-dichlorofluorescein. It is important for fluorescent probe with narrow emission bandwidth to be used in detecting overlapping target substances. For 2',7'-dichlorofluorescein, it has also long wavelength and high fluorescence quantum yield. However, its emission band is wider than 4(7)-chlorofluorescein. Because of this severe constraint, 2',7'-dichlorofluorescein could not be used in detecting multiple target substances in the same sample, and particularly in DNA sequencing systems.

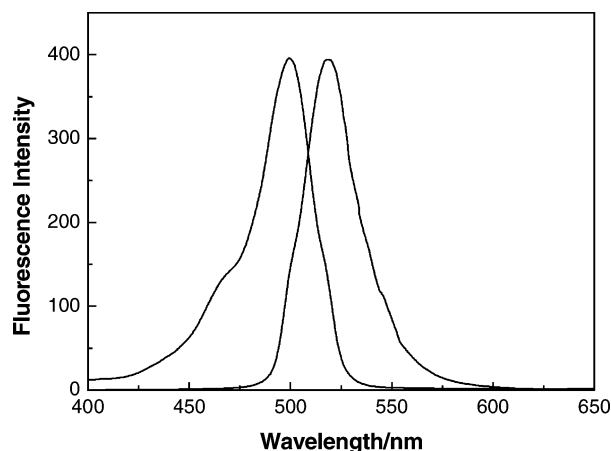


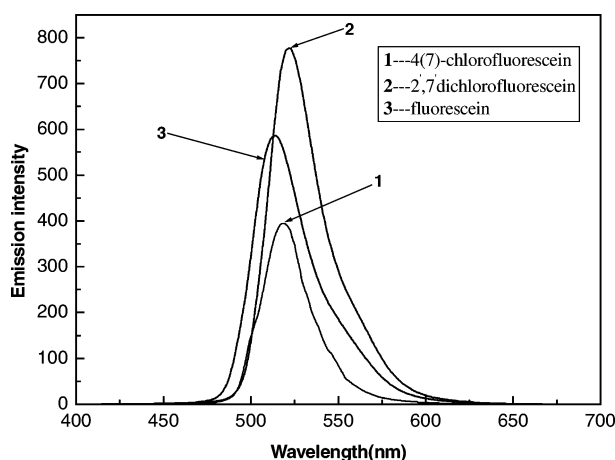
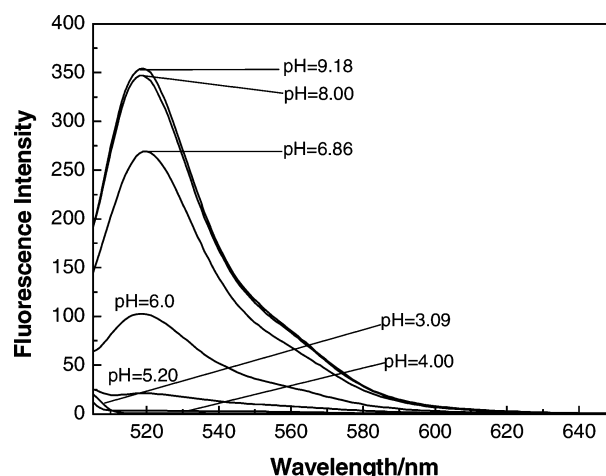
Fig. 6. Excitation and emission spectra of 4(7)-chlorofluorescein.

Table I. Photophysical Properties of Fluorescein, 2',7'-Dichlorofluorescein, and 4(7)-Chlorofluorescein

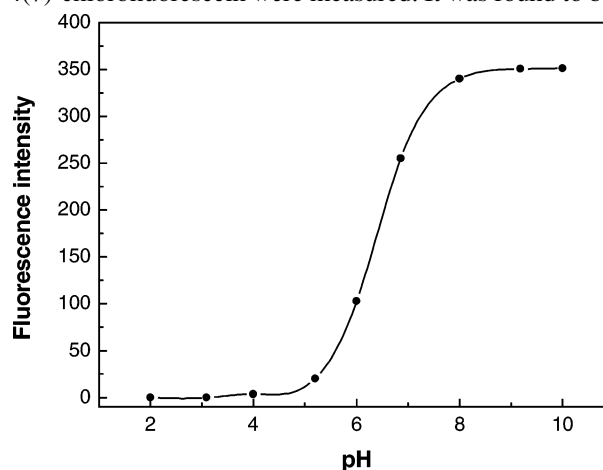
Substrate	Fluorescein	2',7'-Dichlorofluorescein	4(7)-Chlorofluorescein
λ_{ab} (nm)	492	503	499
A	0.0392	0.0515	0.0242
ε (mol/cm L $\times 10^4$)	7.84	10.30	4.84
λ_{ex} (nm)	492	503	499
λ_{em} (nm)	514	522	518
Stokes shift (cm^{-1})	870.0	723.6	734.9
F	586.856	778.040	399.873
ϕ	0.90	0.86	0.93
Emission band halfwidth (nm)	37	35	31

The relationship between the fluorescence intensity and pH were studied in this paper. As shown in Fig. 8, 4(7)-chlorofluorescein showed clear pH-dependence in the fluorescence intensity. Under the condition of $\text{pH} < 4.0$, the fluorescence intensity approaches zero and 4(7)-chlorofluorescein have no fluorescence. With the increase in pH value, fluorescence intensity of 4(7)-chlorofluorescein increases. When pH is 8.0, the fluorescence intensity reaches the maximum. And when pH is also raised, the fluorescence intensity does not change. Therefore, 4(7)-chlorofluorescein are strongly pH-dependent between 4.0 and 8.0 (Fig. 9). By taking advantage of the pH dependence of their fluorescence, 4(7)-chlorofluorescein could be used to follow cell pH and detect cell.

In conclusion, we have described new fluorescent substrates 4- and 7-chlorofluorescein. The ease of synthesis and purification make these reagents more attractive than the traditionally used fluorescent reagents. They were found to have absorption and emission maxima at long wavelength, high fluorescence quantum yield, and

**Fig. 7.** Emission spectra of 4(7)-chlorofluorescein, 2',7'-dichlorofluorescein, and fluorescein.**Fig. 8.** Emission spectra of 4(7)-chlorofluorescein in buffered solution of various pH.

narrow emission bandwidth. These fluorescent properties are highly favorable for them to be used as molecular probes, especially for detecting multiple target substances in the same sample. In addition, the pH dependence of 4(7)-chlorofluorescein were measured. It was found to be

**Fig. 9.** Effect of pH on fluorescence intensity of 4(7)-chlorofluorescein.

strongly pH-dependent between 4.0 and 8.0, and could be used as pH-sensitive probe to detect pH in the cells. We expect 4- and 7-chlorofluorescein will have latent application in many analytical and diagnostic techniques.

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