

## Fluorescence Sensor Performance of a New Fluorescein Derivate: [2-Morpholine-4-(6-chlorine-1,3,5-s-triazine)-amino]fluorescein

Fengyan Ge,<sup>†,\*</sup> Chun Yang,<sup>‡</sup> and Zaisheng Cai<sup>†</sup>

<sup>†</sup>College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China. \*E-mail: fyge@dhu.edu.cn

<sup>‡</sup>Wuhan Product Quality Supervision & Testing Institute, Wuhan 430043, China

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A novel reactive fluorescent dye [2-morpholine-4-(6-chlorine-1,3,5-s-triazine)-amino]fluorescein based on 5-aminofluorescein was synthesized by electrophilic substitution. The photophysical properties, solvent effect, pH value sensitivity, and metal ions responsibility of this new fluorophore were investigated. Compared with 5-aminofluorescein, the novel fluorophore exhibited stronger fluorescence and longer lifetime. The fluorescence property of the new dye was obviously affected by different solvents and pH values, and it showed stronger fluorescence in the protic solvents or an alkaline environment. Moreover, the fluorescent intensity could be enhanced by the formation of complex with metal ions especially with Mg<sup>2+</sup>. The results show that the fluorescent dye is a promising efficient sensor for solvents, protons, and metal ions.

**Keywords:** [2-Morpholine-4-(6-chlorine-1,3,5-s-triazine)-amino]fluorescein, Solvent, pH, Metal ion, Fluorescence sensor

### Introduction

During the past years, fluorescein and its derivatives have been widely employed as molecular probes in chemical biology.<sup>1–8</sup> More attention has been paid to aminofluorescein (AF) because it could specifically combine with the biomolecules at the site of –OH, –SH, and –COOH, which makes it a potential fluorescent probe. However, AF has a low quantum yield of 0.015 and exhibits none of fluorescein's indicator properties which restrict its application. Munkholm reported that AF can recover the fluorescence till the electron lone pair of the amine is unavailable for electron transfer through covalent or electrostatic binding process.<sup>9</sup> According to the work of Munkholm, the reaction between AF and less electrophilic species (for example, triazine chloride or sulfonyl chloride) could recover the fluorescence. As an important bridge molecule and a synthetic intermediate, the triazine chloride has been widely used in the preparation of dyes and fluorescein brightening agents due to the fact that the three chlorine atoms on the triazine ring can be substituted subsequently.

Following the principle mentioned above, we fabricated and synthesized a novel reactive fluorescein derivative [2-morpholine-4-(6-chlorine-1,3,5-s-triazine)-amino]fluorescein, namely DTAF-MOR, by combining 5-AF and morpholine with triazine chloride which is a typical electrophilic species for the fluorescence recovery. Meanwhile, the introduction of triazine chloride brings more reactive sites for combining with biomolecules and textiles, which in turn reinforce the bonding fastness. In addition, an environmental responsible ability was obtained because the morpholine molecule can form complex with metal ions and improve the sensitivity to solvents and pH values.

In this paper, the structure of this novel fluorescein derivative DTAF-MOR was confirmed by <sup>1</sup>H and <sup>13</sup>C NMR spectra, MS (ESI) spectra, and FT-IR spectra. And the photophysical properties, solvent effect, pH value sensitivity, and metal ions responsibility were also investigated.

### Experimental

**Materials.** All solvents were purchased from Sinopharm Chem (Shanghai, China) and used without further purification. To adjust the pH value, very small volumes of hydrochloric acid and sodium hydroxide were used. NiSO<sub>4</sub>·6H<sub>2</sub>O, MnSO<sub>4</sub>·H<sub>2</sub>O, CoSO<sub>4</sub>·7H<sub>2</sub>O, MgSO<sub>4</sub>·7H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, FeSO<sub>4</sub>·7H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, Cd(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, BaCl<sub>2</sub>, AgNO<sub>3</sub>, K<sub>2</sub>SO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, Pd(NO<sub>3</sub>)<sub>2</sub>, and CaCl<sub>2</sub> salts were the sources for metal ions. The effect of metal ions on the fluorescent intensity was examined by continually adding 12 μL metal ions solution (5.0 × 10<sup>-5</sup> mol/L) to 3 mL dye solution (5.0 × 10<sup>-6</sup> mol/L). To avoid the influence of the dilution, the addition was limited to 96 μL.

**Methods.** <sup>1</sup>H and <sup>13</sup>C spectra were recorded on Bruker AM 300 and Bruker AV-400 spectrometer (Billerica, MA, USA), respectively. The measurements were carried out in DMSO-*d*<sub>6</sub> or CDCl<sub>3</sub> solution using tetramethylsilane (TMS) as an internal standard. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Perkin-Elmer 2000 spectrometer (Waltham, MA, USA) using KBr disks. MS was taken on a Varian 310-MS (Palo Alto, CA, USA). UV-Vis spectra were recorded on a HITACHI U-3310 spectrophotometer (Tokyo, Japan) at the room temperature. The fluorescence spectra were examined on a HITACHI F-7000 fluorescence spectrophotometer and fluorescence lifetime

was measured on a PTIQM/TM fluorescence spectrophotometer. The fluorescence quantum yields ( $\Phi_F$ ) were measured relatively to fluorescein ( $\Phi_{ref} = 0.90$ ) according to the available method in the literature.<sup>10</sup> Different pH buffer solutions were prepared by mixing different amounts of hydrochloric acid and sodium hydroxide. All pH measurements were carried out with the pH meter. The effect of the metal ions on the fluorescent intensity was examined by adding stock solution of metal ions to the dye solution.

### Synthesis of 2-Morpholine-4,6-dichloro-1,3,5-s-triazine 3.

Synthesis of intermediate **3** was performed in one step as shown in Figure 1. A solution of morpholine **2** (8.9 mL, 100 mmol) in 38 mL water was added dropwise into the suspension of cyanuric chloride **1** (18.4 g, 100 mmol) in 100 mL acetone at 0 °C. The mixture was stirred at 0 °C for 1 h. A solution of 50 mL  $\text{Na}_2\text{CO}_3$  (6.4 g, 60 mmol) was slowly added to keep an alkaline condition and the reaction was held for 1 h. The mixture was filtered, washed with ice water and dried to give **3** (18.8 g, yield 80%) as a white solid powder.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) ( $\delta$ , ppm): 3.89 (t,  $J = 4.4$  Hz, 4H), 3.76 (t,  $J = 4.4$  Hz, 4H);  $^{13}\text{C}$  NMR (400 MHz,  $\text{CDCl}_3$ ) ( $\delta$ , ppm): 170.46, 164.14, 66.37, 44.47; FT-IR (KBr) ( $\text{cm}^{-1}$ ): 2918.65, 2857.12, 1626.62, 1349.91, 1155.74, 1111.84.

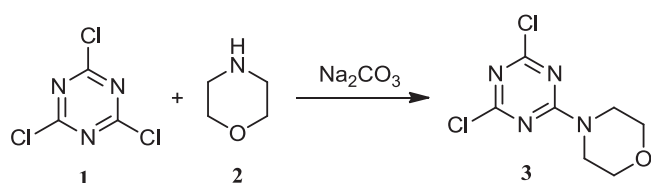


Figure 1. Synthetic route of intermediate **3**.

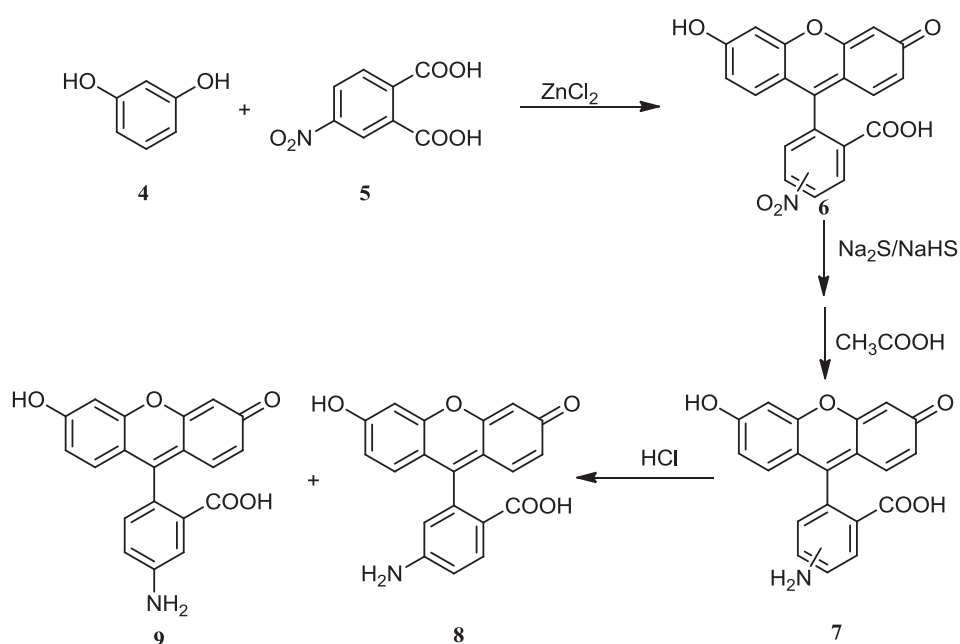


Figure 2. Synthetic route of 5-AF **9**.

**Synthesis of 5-Aminofluorescein 9.** 5-AF **9** was synthesized according to the method as illustrated in Figure 2. 4-nitrophthalic acid **5** (10 g, 45 mmol), resorcinol **4** (12.44 g, 113 mmol), and zinc chloride (13.62 g, 100 mmol) were heated at 120 °C for melting. The mixture was stirred at 150 °C for 2 h until the material solidified. The mixture was pulverized and boiled in 150 mL 1 M HCl for 1 h. The solid was collected on a frit, washed several times with hot water, and dried in vacuo to obtain an orange precipitate 5(6)-nitrofluorescein **6**.

5(6)-nitrofluorescein **6** (7.6 g, 20 mmol), sodium hydrosulfide (8.2 g, 146 mmol) and 250 mL sodium sulfide (17.5 g, 73 mmol) solution were mixed and refluxed for 24 h. The solution was cooled down to the room temperature and a red–brown precipitate was collected by adding acetic acid to the solution. The precipitate was refluxed for 6 h with 300 mL diluted hydrochloric acid and filtered to get rid of the sulfur. The filtrate was cooled at 4 °C for 24 h to obtain 5-AF. The crude was purified by column chromatography on silica gel ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$ , 1:15) to give **9** (2.94 g, yield 40%).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ) ( $\delta$ , ppm): 10.04 (s, 2H), 6.97 (s, 1H), 6.94 (d,  $J = 8.3$  Hz, 1H), 6.84 (d,  $J = 8.1$  Hz, 1H), 6.63 (s, 2H), 6.58 (d,  $J = 8.4$  Hz, 2H), 6.53 (d,  $J = 8.6$  Hz, 2H), 5.72 (s, 2H);  $^{13}\text{C}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ) ( $\delta$ , ppm): 170.20, 159.56, 152.39, 150.72, 139.85, 129.35, 127.79, 124.55, 122.47, 112.94, 111.07, 106.89, 102.61, 83.28; FT-IR (KBr) ( $\text{cm}^{-1}$ ): 3424.24, 1715.09, 1601.22, 1467.70, 1388.70, 1318.80, 1274.35, 1174.71, 849.48; MS (ESI):  $m/z = 345.9$  ( $\text{M}-\text{H}^-$ ), calcd. 347.08.

**Synthesis of DTAF-MOR 10.** DTAF-MOR **10** was synthesized according to the method as illustrated in Figure 3. A mixed solution of intermediate **3** (0.48 g, 2 mmol) and 5-AF **9** (0.7 g, 2 mmol) in EtOH was heated to 40 °C and stirred

for 2 h. A solution of 6 mL  $\text{Na}_2\text{CO}_3$  (0.11 g, 1 mmol) was added dropwise into the solution to keep an alkaline condition. The reaction was maintained for about 3 h and the completion was monitored by TLC (thin layer chromatography). The solution was concentrated and dried to give orange-red powder. The crude was purified by column chromatography on silica gel ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$ , 1:15) to give DTAF-MOR **10** (0.43 g, yield 38%).

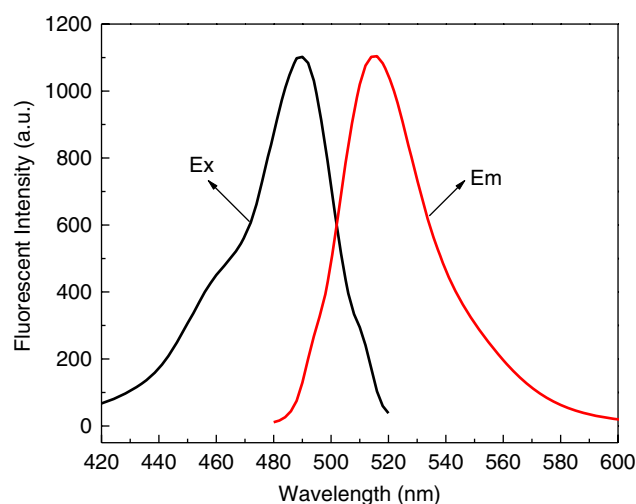
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ) ( $\delta$ , ppm): 10.61 (s, 1H), 10.14 (s, 2H), 8.32 (s, 1H), 8.02 (s, 1H), 7.23 (d,  $J=8.4$ , 1H), 6.68 (d,  $J=1.8$ , 2H), 6.62 (d,  $J=8.7$ , 2H), 6.56 (dd,  $J=8.7$ , 2.1, 2H), 3.78 (d,  $J=16.3$ , 4H), 3.68 (s, 4H);  $^{13}\text{C}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ) ( $\delta$ , ppm): 168.91, 164.46, 164.04, 159.70, 152.27, 146.97, 140.89, 137.58, 129.55, 127.30, 124.77, 112.99, 110.08, 102.60, 83.49, 72.69, 66.18, 60.66, 44.28; FT-IR (KBr) ( $\text{cm}^{-1}$ ): 3441.04, 2858.19, 1752.58, 1577.40, 1385.83, 1239.24, 1168.92, 1239.24, 1108.88, 847.11, 797.49; MS (ESI):  $m/z=543.9$  ( $\text{M}-\text{H}$ ) $^-$ , calcd. 545.11.

## Results and Discussion

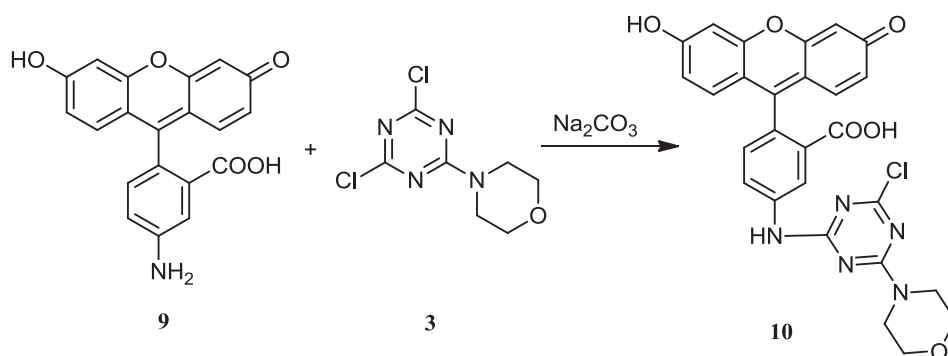
**Photophysical Properties of DTAF-MOR.** The fluorescent properties of DTAF-MOR were investigated by comparison with 5-AF. The wavelength of absorption maxima ( $\lambda_{\text{ab}}$ ), the wavelength of excitation maxima ( $\lambda_{\text{ex}}$ ), the wavelength of emission maxima ( $\lambda_{\text{em}}$ ), extinction coefficient ( $\epsilon$ ), Stokes shift, fluorescence quantum yield ( $\Phi_{\text{F}}$ ), and lifetime ( $\tau$ ) were summarized in Table 1. As can be seen from Table 1, the maxima absorption wavelength of DTAF-MOR was 490 nm which was similar with that of 5-AF (488 nm). The fluorescence quantum yield of DTAF-MOR was 0.294 which was higher than that of 5-AF (0.017). This attributed to the electron-withdrawing effect of s-triazine. Compared with 5-AF, DTAF-MOR had a larger Stokes shift and longer lifetime.

Moreover, the excitation and emission spectra were of typical mirror symmetry as shown in Figure 4.

**Influence of Solvents on the Fluorescent Intensity of DTAF-MOR.** Solvent polarity and viscosity considerably impacts the fluorescent intensities of fluorescent compounds.<sup>11</sup> The effect of solvents on the fluorescent intensity of DTAF-MOR and 5-AF was investigated. The results were displayed in Figure 5. DTAF-MOR exhibited an enhanced fluorescence in the protic solvents, such as alcohols and water. However, such a fluorescent enhancement did not appear in the aprotic solvents such as THF, DCM, ACE, and other solvents. The similar phenomena were also reported by Reichardt<sup>12</sup> who found when the solvent polarity empirical parameter  $E_{\text{T}}$  value of the solvent was larger than 50, the enhanced fluorescence could be observed, otherwise, the fluorescence would vanish. DTAF-MOR possessed a



**Figure 4.** Excitation and emission spectra of DTAF-MOR in 0.1 N NaOH.

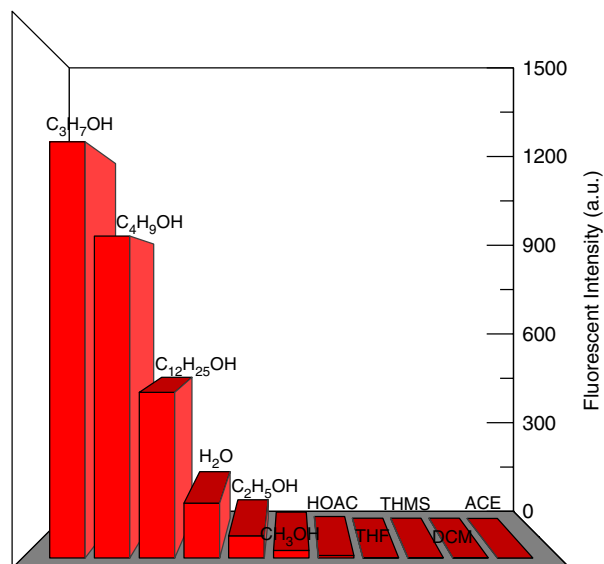


**Figure 3.** Synthetic route of DTAF-MOR **10**.

**Table 1.** Photophysical properties of 5-AF and DTAF-MOR.

Compound	$\lambda_{\text{ab}}$ (nm)	$\lambda_{\text{ex}}$ (nm)	$\lambda_{\text{em}}$ (nm)	$\epsilon$ ( $\times 10^4$ mol/cm $^{-1}$ L)	Stokes shift ( $\text{cm}^{-1}$ )	$\Phi_{\text{F}}$	$\tau$ (ns)
5-AF	488	519	538	7.94	680.46	0.017	0.923
DTAF-MOR	490	489	515	2.74	1032.40	0.294	1.944

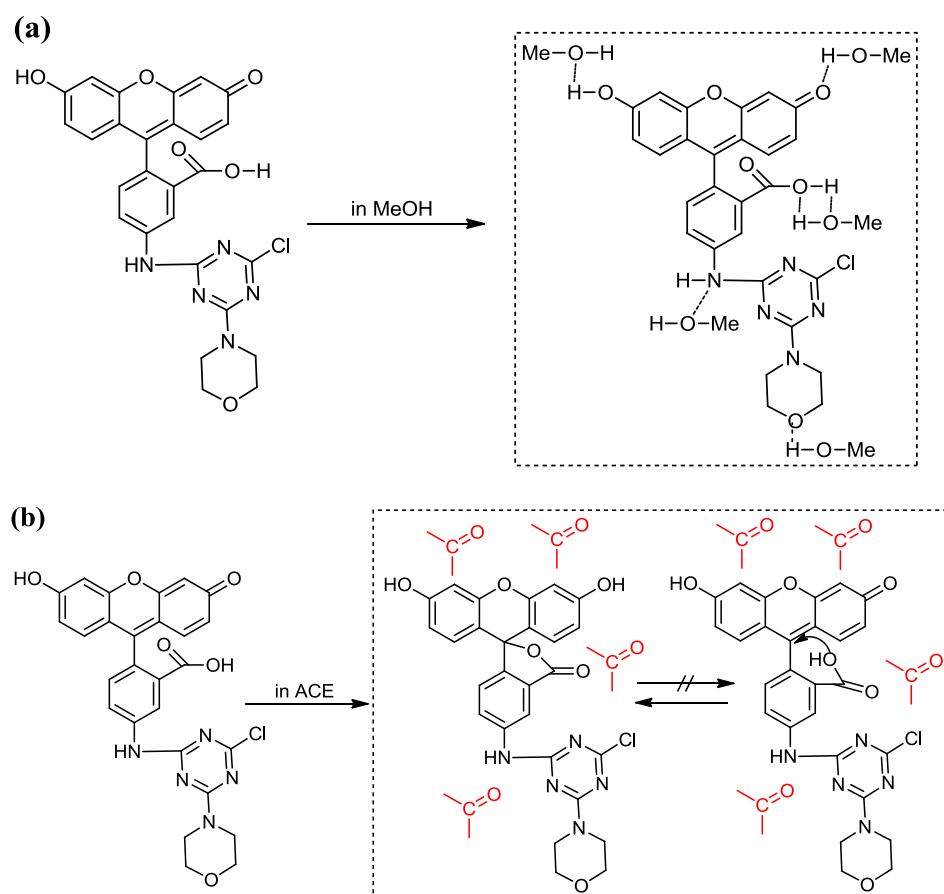
solvatochromic-responsive characteristics toward the protic solvents and the aprotic solvents which may become a promising effective indicator for solvents. The phenomena



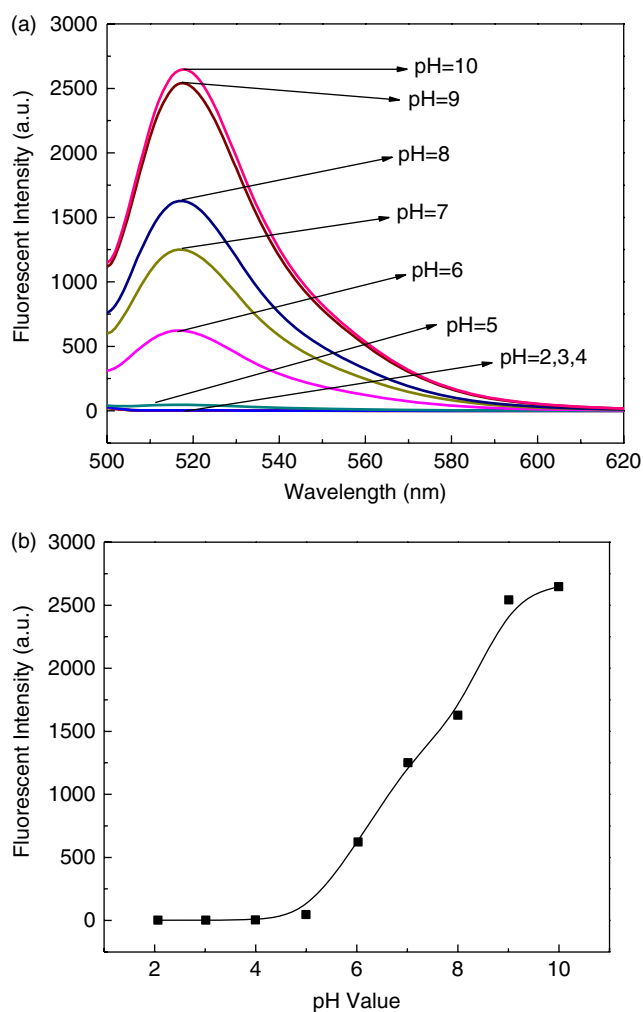
**Figure 5.** Fluorescent intensities of DTAF-MOR in different solvents.

observed above might be explained by the mechanism as shown in Figure 6. DTAF-MOR can be effectively encapsulated into methanol through the intermolecular interactions between the nitrogen or oxygen atoms in the DTAF-MOR molecules and hydroxyl groups in methanol. Especially, the hydrogen bonding interactions would play an important role in stabilizing quinoid-type of DTAF-MOR.<sup>13</sup> The fluorescent intensities in alcohols were increased with an increase of  $E_T$  value of alcohols as follows:  $n$ -BuOH $\approx n$ -PrOH $>$ EtOH $>$ MeOH. This result would attribute to the protonation ability of alcohols. On the other hand, the hydrogen bonding interactions between the dye and the aprotic solvents such as ACE would not be formed. The quinoid-type of DTAF-MOR would become unstable in ACE, and DTAF-MOR existed in the lactone type without fluorescence.

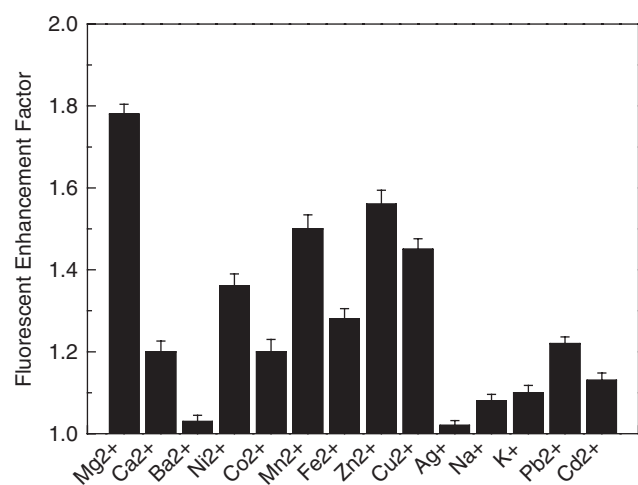
**Influence of pH Value on the Fluorescent Intensity of DTAF-MOR.** The fluorescence pH titrations were carried out in the pH values ranging from 2 to 10. As shown in Figure 7, in solutions below pH 4, the fluorescent intensity approached zero and DTAF-MOR had no fluorescence. An increase in the pH value led to an enhancement in the fluorescent intensity of DTAF-MOR. When pH value was 9, the fluorescent intensity reached the maximum. And further



**Figure 6.** (a) Schematic illustration for the interaction between DTAF-MOR and MeOH; (b) Schematic illustration for the interaction between DTAF-MOR and ACE.



**Figure 7.** (a) Changes in the fluorescence spectra of DTAF-MOR as a function of pH; (b) Effect of the fluorescent intensity of DTAF-MOR on the pH.



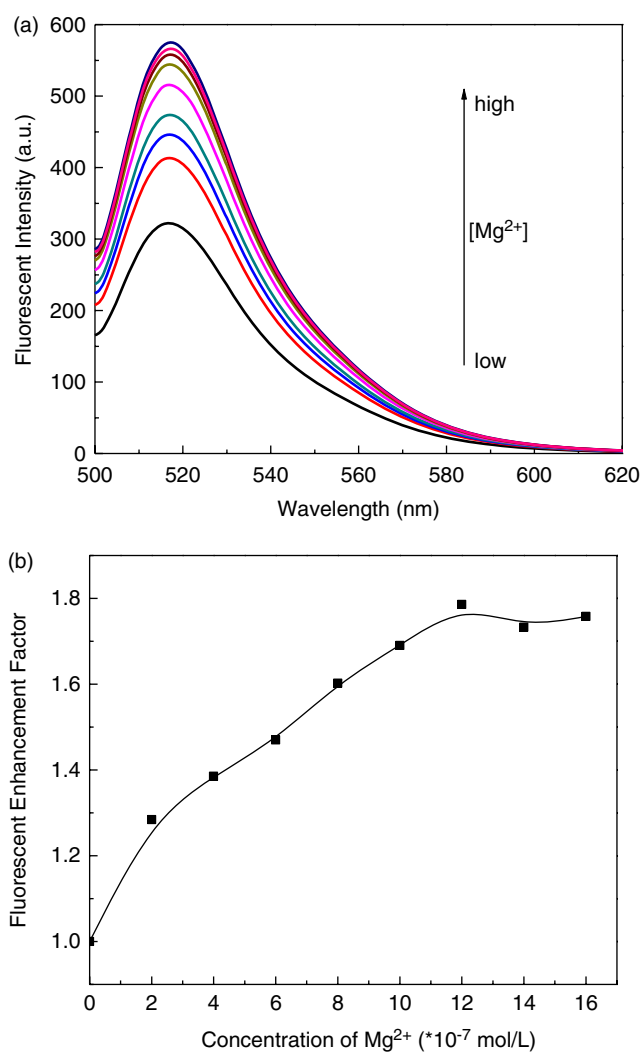
**Figure 8.** Fluorescence enhancement factor of DTAF-MOR ( $5.0 \times 10^{-6}$  mol/L) toward different metal ions.

increasing the pH value, it is not beneficial for the fluorescent enhancement. Therefore, it can be concluded that DTAF-MOR is sensitive to the pH values ranging from 4 to 9. In order to accurately determine pH value, the  $pK_a$  value was calculated according to the Eq. (1):

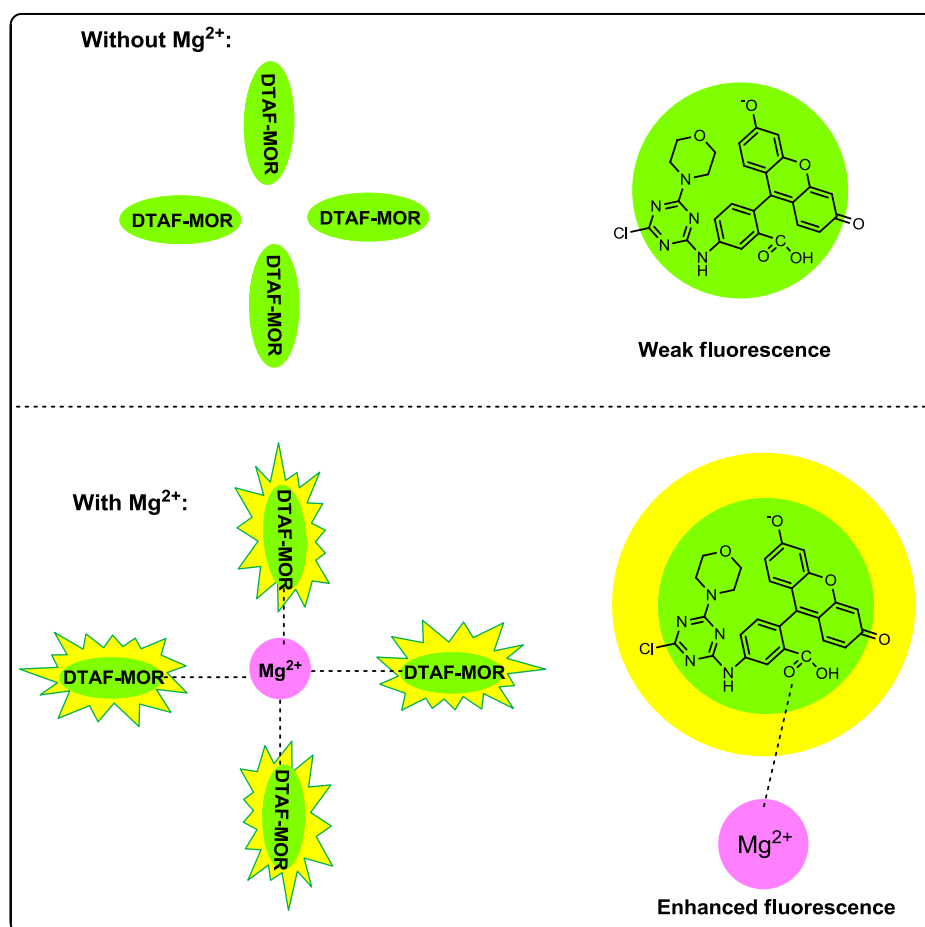
$$\log \left[ \frac{(IF_{\max} - IF)}{(IF - IF_{\min})} \right] = pH - pK_a \quad (1)$$

Based on Eq. (1), the  $pK_a$  value of DTAF-MOR was found to be at 6.94. The  $pK_a$  value of 5-AF was reported to be 3.90.<sup>9</sup> This is because of the strong electron-donating ability of morpholine group in DTAF-MOR structure. The  $pK_a$  value indicated that DTAF-MOR would be very suitable for monitoring changes in the physiological pH range.

The fluorescent property of fluorescent dyes can be enhanced by the formation of complex with metal ions.<sup>14–16</sup> The fluorescent properties of DTAF-MOR in the presence



**Figure 9.** (a) Fluorescence spectra of DTAF-MOR at the various concentrations of  $Mg^{2+}$ ; (b) Fluorescent enhancement factor of DTAF-MOR toward  $Mg^{2+}$  with the different concentrations.



**Figure 10.** Schematic representation of complexation mode of DTAF-MOR with  $\text{Mg}^{2+}$  ions.

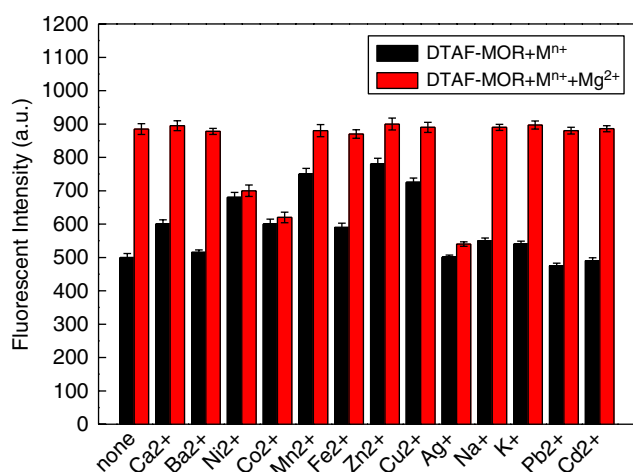
of various metal ions were investigated in DMF with regard to its potential application as a sensor. The enhancement factor  $FE = I/I_0$  was calculated by dividing the fluorescent intensities maximal ( $I$ ) by minimal ( $I_0$ ) fluorescent intensities recorded after and before addition of metal ions. The influence of different metal ions ( $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Pd}^{2+}$ , and  $\text{Ca}^{2+}$ ) on the fluorescence behavior of DTAF-MOR were presented in Figure 8. The results showed that the different metal ions can increase the fluorescent intensities of DTAF-MOR and the highest fluorescence enhancement factor of the dye was observed in the presence of  $\text{Mg}^{2+}$  (enhancement factor = 1.786). The sensor capacity of DTAF-MOR ( $5 \times 10^{-6}$  mol/L) at different concentrations of  $\text{Mg}^{2+}$  was displayed in Figure 9. An increase in the fluorescent intensity was found after addition of  $\text{Mg}^{2+}$  in the concentration range of  $2 \times 10^{-7}$ – $1.6 \times 10^{-6}$  mol/L. And the maximum enhancement was observed at  $\text{Mg}^{2+}$  concentration of  $1.2 \times 10^{-6}$  mol/L. Further increasing  $\text{Mg}^{2+}$  concentration had only a slight impact on the fluorescent intensity. The fluorescence enhancement was due to the complexation between carbonyl group and  $\text{Mg}^{2+}$ , as shown in Figure 10. By calculating, a 1:4 metal/ligand complex was obtained.

In addition, the selectivity of DTAF-MOR toward  $\text{Mg}^{2+}$  in the presence of other metal ions was investigated in Figure 11.

Relatively low interference levels were found for detection of  $\text{Mg}^{2+}$  in the presence of  $\text{Cd}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Pd}^{2+}$ . The DTAF-MOR responses for  $\text{Mg}^{2+}$  in the coexistence of  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Ag}^+$  were relatively low. The above mentioned experiments indicated that DTAF-MOR can be used for the analysis of  $\text{Mg}^{2+}$  in biological concerns and environment field.

## Conclusion

In this paper, we have designed the DTAF-MOR, containing s-triazine and morpholine moieties. Compared with 5-AF, the new dye displays stronger fluorescence, larger Stokes shift, and a longer lifetime. DTAF-MOR has higher fluorescence quantum yield 0.294 than 5-AF 0.015 due to the electron-withdrawing effect of s-triazine. DTAF-MOR possesses a solvatochromic-responsive characteristics toward protic and aprotic solvents and the hydrogen bonding interactions would play an important role in stabilizing quinoid-type of the dye. DTAF-MOR is sensitive to the pH values ranging from 4 to 9 and its  $\text{pK}_a$  value is higher 3  $\text{pK}_a$  units than that of 5-AF due to the electron-donating morpholine group. In the presence of metal ions ( $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,



**Figure 11.** Selectivity of DTAF-MOR ( $5.0 \times 10^{-6}$  mol/L) toward different metal ions (1.0 equiv) in the absence and presence of  $\text{Mg}^{2+}$  (1.0 equiv). The black bars represent the fluorescent intensity of the solution with DTAF-MOR and different metal ions; the red bars represent the fluorescence changes upon addition of  $\text{Mg}^{2+}$  to the solution containing DTAF-MOR and other metal ions.

$\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ag}^{+}$ ,  $\text{K}^{+}$ ,  $\text{Na}^{+}$ ,  $\text{Pd}^{2+}$ , and  $\text{Ca}^{2+}$ ), especially  $\text{Mg}^{2+}$ , the fluorescent intensities of DTAF-MOR can be enhanced. The results show that the novel dye is a promising efficient “off–on” fluorescence sensor for solvents, pH value, and metal ions.

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**Supporting Information.** Additional supporting information is available in the online version of this article.

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