

A Novel Small Molecule Fluorescent Sensor for Zn²⁺ Based on Pyridine-Pyridone Scaffold

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Abstract

The development of a water-soluble and small molecular weight fluorescent probe, 3-(4-methoxyphenyl)-4-(methylsulfanyl)-6-(pyridin-2-yl)pyridin-2(1H)-one (**3**), for detecting Zn²⁺ based on pyridine-pyridone skeleton is reported. We observed a clear chelation enhanced fluorescence effect of **3** in the presence of Zn²⁺. Other fluorescent properties of **3** are discussed.

Keywords

Zn²⁺, Fluorescent sensor, Pyridine-pyridone, Small molecule, Water-soluble

1. Introduction

Zinc is an essential element for life and is known to play important roles in biological processes including gene expression, apoptosis, enzyme regulation, immune system and neurotransmission [1–6]. Most of Zn²⁺ in living cells are bound to the proteins, which are called as metalloprotein [7–9]. Zinc metalloproteases employ Zn²⁺ as the catalysis center and a most well-known example in this class is angiotensin converting enzyme (ACE) [10–12]. Zinc chelation also contributes to the structure stabilization of proteins. For example, the zinc finger proteins form a stable three dimensional structure to interact a specific DNA sequence for controlling gene functions [13].

There also are free or chelatable Zn^{2+} in living cells, which may be involved in both physiology and disease states. In pancreatic β -cells, Zn^{2+} participates in controlling synthesis, storage and secretion of insulin. When Zn^{2+} is short, blood sugar level rises by a secretion delay of the insulin [14,15]. In central nervous system, free or chelatable Zn^{2+} are co-localized with glutamate in presynaptic vesicles of the mammalian hippocampus [16,17].

To investigate physiological roles of free or chelatable Zn^{2+} in living cells, we should know their concentration in each tissue and in different (patho-) physiological states. In this context, a number of fluorescent sensors have recently been developed based on elegant ideas [18–20]. Although they contributed great extends to understand Zn^{2+} roles in physiology and particularly in the field of neurochemistry [16,17], its mechanisms of action is not well understood in comparison with other cations such as Na^+ , K^+ , Ca^{2+} , etc.

We realized that Zn^{2+} selective fluorescent probes have mainly used three core structures, namely quinoline, BF_2 chelated dipyrromethene and fluorescein [21–30]. After modification of the core structure in getting selectivity toward Zn^{2+} , the molecule obviously became bigger and more hydrophobic. Therefore most of the fluorescent probes prepared so far have undesirable characteristics in terms of aqueous solubility. This problem should be overcome somehow to develop a new simple fluorescent probe based upon different chemical backbone structures. We thought that if we could find a new and small molecular weight (MW = ca. 300) fluorescent core structure having certain selectivity toward Zn^{2+} , such molecules would become an advantageous starting point for designing a new fluorescent sensor. If the initial core structure is small enough, the fluorescent probes may still be molecular weight below 500 with desirable physico-chemical properties, even after the modifications [31]. We have recently published a new and simple synthetic method for preparing pyridine-pyridone derivative **3** [32]. Since then we have been interested in the molecule characters of this class of heterocyclic compounds. In this paper, we report the fluorescent characteristics of pyridine-pyridone derivative **3**, MW = 324, that can potentially be used as a lead structure for finding a new fluorescent sensor for Zn^{2+} .

2. Experimental

2.1. Materials and instruments

All the solvents were of analytic grade and used as received. The solutions of metal ions were prepared from NaCl, KCl, $BaCl_2$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnCl_2$, $CdCl_2 \cdot 2.5H_2O$, $CuCl_2$, $MnCl_2 \cdot 4H_2O$, $AlCl_3 \cdot 6H_2O$, respectively, and were dissolved in distilled water. 1H NMR was measured on a JEOL-GX-400 (400MHz) with chemical shifts reported as ppm (in

DMSO-*d*₆). HRMS was measured on a JMS-T100LP mass spectrometer. Mass spectra (MS) were recorded on a JEOL-DX-303 mass spectrometer. Fluorescence spectra were determined on a Jasco FP-6200 spectrofluorometer. Ultraviolet (UV) absorption spectra were determined in 95% ethanol on a Hitachi 323 spectrometer. Infrared (IR) spectra were recorded in potassium bromide pellets on JASCO 810.

2.2. Synthesis of 3-(4-methoxyphenyl)-4-(methylsulfanyl)-6-(pyridin-2-yl)pyridin-2(1H)-one (**3**)

Powdered sodium hydroxide (0.40 g, 10.0 mmol) was added to a solution of 1.13 g (5.0 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone (**1**) [32] and 0.88 g (6.0 mmol) of **2** in 50 mL of DMSO, and the mixture was stirred for 2 h at room temperature. The reaction was poured into 300 mL of ice water and neutralized with a 10% hydrochloric acid solution. The mixture was extracted with 100 mL of dichloromethane three times. The combined organic extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue and a 1% hydrochloric acid solution was refluxed for 1 h. After evaporation, the residual solid was recrystallized from methanol to give **3** (1.01 g, 62%) as yellow leaflets. Mp: 260-261 °C. IR (KBr, cm⁻¹): 3456, 1605, 1510, 1275, 1175. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 2.48 (s, 3H), 3.80 (s, 3H), 6.96 (d, *J* = 8.8 Hz, 2H), 7.20 (d, *J* = 8.8 Hz, 2H), 7.50 (ddd, *J* = 2.9, 4.9, 7.8 Hz, 1H), 7.97 (ddd, *J* = 1.5, 2.0, 7.8 Hz, 1H), 8.25 (d, *J* = 7.8 Hz, 1H), 8.25 (d, *J* = 7.8 Hz, 1H), 8.72 (ddd, *J* = 1.0, 3.9, 4.9 Hz, 1H), 11.09 (brs, 1H). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 14.46, 55.06, 113.41, 120.93, 124.73, 127.09, 131.35, 137.63, 149.41, 151.54, 158.62, 159.71. Ms m/z: 325 (M⁺+1, 12), 324 (M⁺, 36), 310 (11), 309 (42), 294 (7), 278 (10), 149 (11), 84 (19), 66 (21), 57 (11), 44 (100). Anal. Calcd. for C₁₈H₁₆N₂O₂S: C, 66.64; H, 4.97; N, 8.64%. Found: C, 66.51; H, 5.07; N, 8.51%. HRMS calcd. for C₁₈H₁₆N₂O₂S: 324.0932; found: 324.0949.

2.3. Synthesis of 6-methoxy-5-(4-methoxyphenyl)-4-(methylsulfanyl)-2,2'-bipyridine (**4**)

Powdered sodium hydroxide (0.20 g, 5.0 mmol) was added to a solution of 0.56 g (2.5 mmol) of **1** and 0.37 g (2.5 mmol) of **2** in 25 mL of DMSO, and the mixture was stirred for 2 h at room temperature. The reaction was poured into 300 mL of ice water and neutralized with a 10% hydrochloric acid solution. The mixture was extracted with 100 mL of dichloromethane three times. The combined organic extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue in 100 mL of methanol was refluxed for 6 h. After evaporation, the residual solid was recrystallized from methanol to give **4** (0.38 g, 45%) as yellow leaflets. Mp: 149-150 °C. IR (KBr, cm⁻¹): 3425, 2940, 1610, 1570, 1540, 1510, 1450, 1430, 1350, 1270, 1250, 1170, 1105, 1035. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 2.47 (s, 3H), 3.81 (s, 3H), 3.90 (s, 3H), 7.00 (d, *J* = 7.8 Hz, 2H), 7.20 (d, *J* = 8.3 Hz, 2H), 7.45 (dd, *J* = 5.4, 6.3 Hz, 1H), 7.95 (dd, *J* = 7.3, 8.3 Hz, 2H), 8.41 (d, *J* = 7.8 Hz, 1H), 8.71 (d, *J* = 4.4 Hz, 1H). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 14.18, 53.35,

55.07, 108.54, 113.77, 120.57, 120.67, 124.18, 126.03, 131.14, 137.32, 149.22, 151.00, 152.19, 154.74, 158.85, 159.90. Ms m/z : 339 ($M^+ + 1$, 24), 338 (M^+ , 100), 337 (29), 323 (19), 293 (8), 291 (7), 161 (6), 121 (5), 105 (5), 44 (4). Anal. Calcd. for $C_{19}H_{18}N_2SO_2$: C, 67.43; H, 5.36; N, 8.28%. Found: C, 67.61; H, 5.50; N, 8.10%. HRMS calcd. for $C_{19}H_{18}N_2O_2S$: 338.1089; found: 338.1098.

2.4. Synthesis of 3-(4-methoxyphenyl)-4-(methylsulfanyl)-6-phenylpyridin-2(1H)-one (**6**)

Powdered sodium hydroxide (0.40 g, 10.0 mmol) was added to a solution of 1.13 g (5.0 mmol) of 3,3-bis-methylsulfanyl-1-phenyl-propenone (**5**) and 0.88 g (6.0 mmol) of **2** in 50 mL of DMSO, and the mixture was stirred for 2 h at room temperature. The reaction was poured into 300 mL of ice water and neutralized with a 10% hydrochloric acid solution. The mixture was extracted with 100 mL of dichloromethane three times. The combined organic extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue and a 1% hydrochloric acid solution was refluxed for 1 h. After evaporation, the residual solid was recrystallized from methanol to give **6** (0.38 g, 24%) as yellow leaflets. Mp: 238-240 °C. IR (KBr, cm^{-1}): 3056, 1568, 1527, 1285, 1176. 1H NMR (DMSO- d_6 , 400 MHz) δ 2.45 (s, 3H), 3.79 (s, 3H), 6.56 (s, 1H), 6.95 (d, $J = 8.8$ Hz, 2H), 7.18 (d, $J = 8.8$ Hz, 2H), 7.49 (m, 3H), 7.82 (dd, $J = 3.9, 7.8$ Hz, 2H), 11.75 (brs, 1H). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 14.31, 55.04, 101.25, 113.37, 123.56, 127.04, 127.34, 128.74, 129.72, 131.44, 133.78, 151.43, 158.50, 160.63. HRMS calcd. for $C_{19}H_{17}NO_2S$: 323.0980; found: 323.0940.

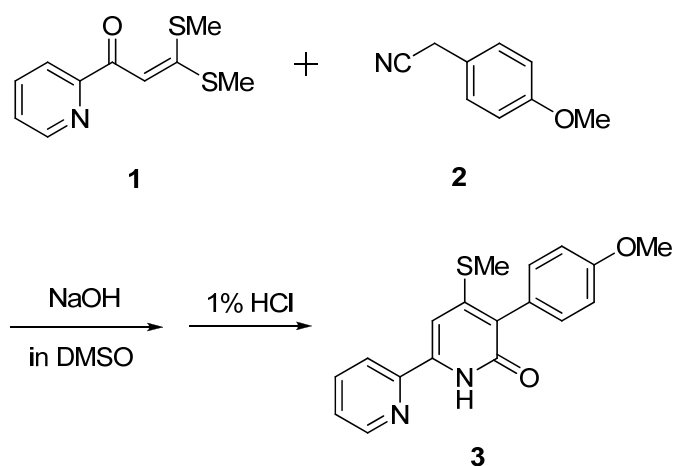
2.5. Spectral measurement

The compounds **3**, **4** and **6** stock solution (1×10^{-2} M) was prepared by directly dissolving in DMSO. The UV/vis spectrum of **3** (10^{-4} M) was measured in HEPES buffer (100 mM, 5% DMSO, pH = 7.4), as shown in Fig.1. For the fluorescence analysis, **3** (10^{-6} M) upon addition of Zn^{2+} in the form of perchlorate salt was measured in HEPES buffer (100 mM, 5% DMSO, pH 7.4). The binding stoichiometry of **3** to Zn^{2+} was investigated by Job's plot. We measured the fluorescence intensity of **3** in the following buffers: 100 mM glycine - HCl buffer (pH 2.0), 100 mM citrate buffer (pH 3.0), 100 mM acetate buffer (pH 4.0 – 5.0), 100 mM Phosphate buffer (pH 6.0), 100 mM HEPES buffer (pH 7.0 – 8.0), 100 mM tris-HCl buffer (pH 9.0). The dissociation constant (K_d) of **3** in HEPES buffer was determined by plotting the fluorescence intensity to free Zn^{2+} concentration. The metal selectivity of **3** was investigated in HEPES buffer (100 mM, 5% DMSO, pH 7.4) and that of **4** and **6** were investigated in aqueous EtOH solution (1:1 EtOH/water (v/v)). The cational solutions were prepared from NaCl, KCl, BaCl₂, MgCl₂·6H₂O, CaCl₂, FeCl₃·6H₂O, CoCl₂·6H₂O, NiCl₂·6H₂O, ZnCl₂, CdCl₂·2.5H₂O, CuCl₂, MnCl₂·4H₂O, AlCl₃·6H₂O (10^{-3} M), respectively. The measurements were carried out at 298 K. The

fluorescence quantum yield values were measured with respect to quinine sulfate solution ($\Phi = 0.54$) as standard.

3. Results and discussion

The synthesis of **3** is described in Scheme 1. The reaction of 3,3-bis-methylsulfonyl-1-pyridin-2-yl-propenone (**1**) with 2-(4-methoxyphenyl)acetonitrile (**2**) in the presence of powdered sodium hydroxide in DMSO followed by treatment with 1% HCl gave 3-(4-methoxyphenyl)-4-(methylsulfonyl)-6-(pyridin-2-yl)pyridin-2(1H)-one (**3**) in 62% yield [32]. The structure was established by $^1\text{H}/^{13}\text{C}$ NMR, MS and elemental analysis.



Scheme 1. Synthesis of compound **3**.

Fig. 1 shows the UV/vis spectrum of **3** in HEPES buffer (100 mM, 5% DMSO, pH 7.4). Zn^{2+} addition changed the UV/vis spectrum of **3** in a Zn^{2+} concentration dependent manner. Namely the absorption peaks at 310 nm and 342 nm decreased and contrary those at 350 nm increased after addition of Zn^{2+} . The fluorescence titration of **3** with Zn^{2+} in HEPES buffer (100 mM, 5% DMSO, pH 7.4) was shown in Fig. 2 (excitation wavelength: 366 nm, emission wavelength: 490 nm). Compound **3** showed fluorescence enhancement upon addition of Zn^{2+} without spectral changes. From the resulting titration data, the dissociation constant was calculated : $K_d = 7 \times 10^{-6}$ M. The fluorescence quantum yield (Φ) was 0.25 without Zn^{2+} and was increased to 0.73 by Zn^{2+} addition. Binding analysis using Job's plot between **3** and Zn^{2+} indicated that the complex formation is 1:1 stoichiometry (Fig. 3). Next, we investigated the effect of pH on the fluorescence properties of **3**. The fluorescence spectra of **3** at

various pHs in the absence/presence of Zn^{2+} are shown in Fig. 4. When the solution pH was decreased from pH 9.0 to pH 2.0, the fluorescence intensity of **3** decreased in both absence (a) and presence (b) of Zn^{2+} without major spectral changes. Strong fluorescence was observed within a few seconds after Zn^{2+} addition, indicating that the complex formation should be spontaneous between **3** and Zn^{2+} . At this point, we have recognized that **3** would be a candidate of a new Zn^{2+} fluorescent core structure that we were looking for.

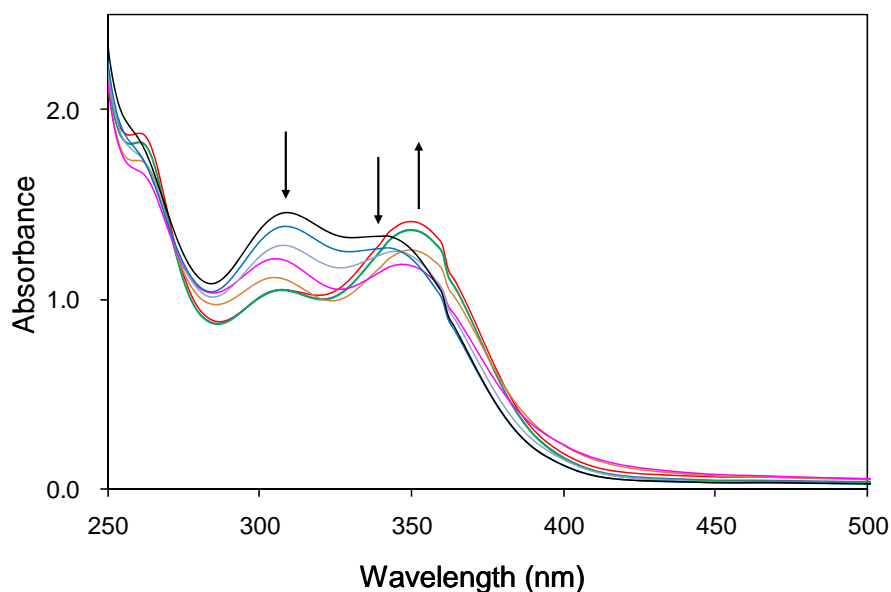


Fig. 1. Absorbance spectra of **3** (10^{-4} M) in HEPES buffer (100 mM, 5% DMSO, pH 7.4) in the presence of Zn^{2+} in the form of perchlorate salt (1×10^{-5} , 5×10^{-5} , 1×10^{-4} , 5×10^{-4} , 1×10^{-3} , 5×10^{-3} , 1×10^{-2} M).

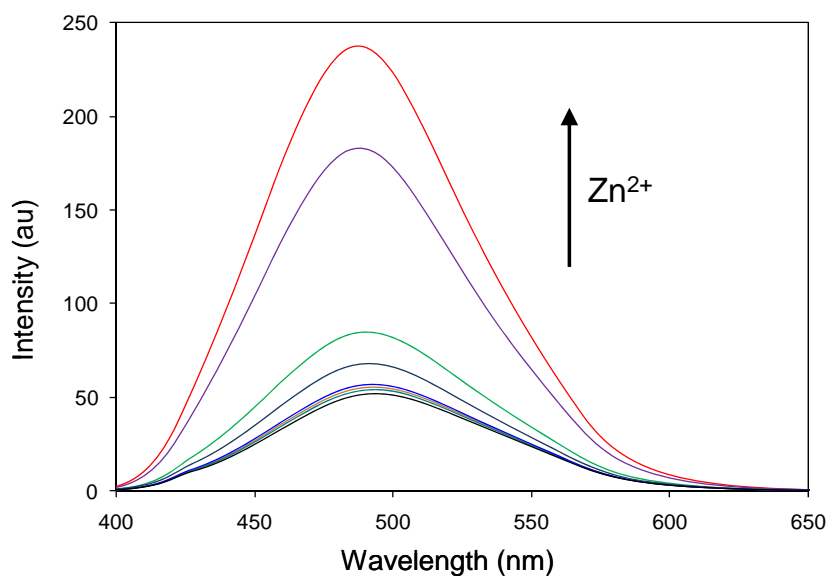


Fig. 2. Fluorescence response of **3** (10^{-6} M) upon addition of Zn^{2+} in the form of perchlorate salt (0, 1, 2.5, 5, 10, 50, 100 μM) in HEPES buffer (100 mM, 5% DMSO, pH 7.4) (excitation wavelength: 366 nm, emission wavelength: 490 nm).

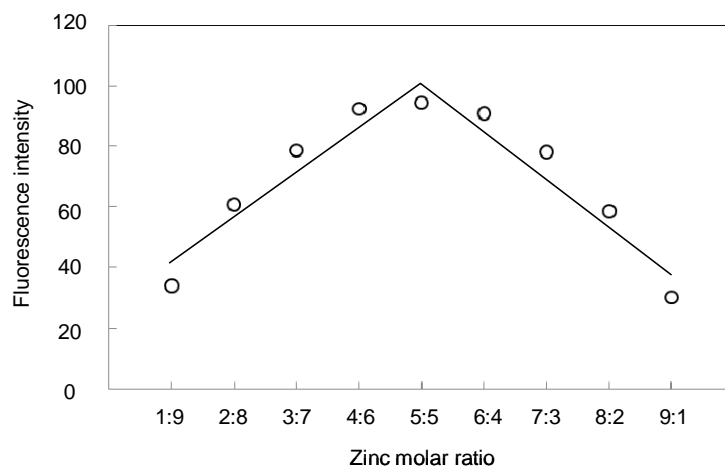


Fig. 3. Job's plot of compound **3** and Zn^{2+} . The total concentrations of compound **3** and Zn^{2+} are 10 μM in HEPES buffer (100 mM, 5% DMSO, pH 7.4) (excitation wavelength: 366 nm, emission wavelength: 490 nm).

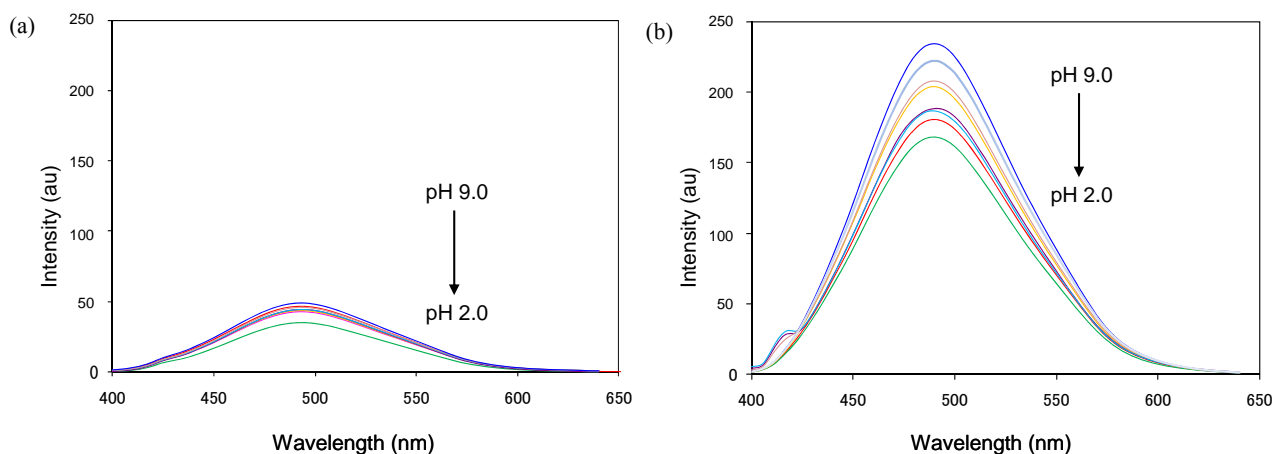


Fig. 4. Effect of pH on the fluorescence properties of (a) compound **3** (10^{-6} M) and (b) compound **3** (10^{-6} M) + Zn^{2+} (1 μM).

We then examined selectivity toward other cations (Al^{3+} , Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , K^+ , Mg^{2+} , Mn^{2+} , Na^+ , Ni^{2+}) in HEPES buffer (100 mM, 5% DMSO, pH 7.4) in Fig. 5. We measured excitation wavelength at 366 nm and emission wavelength at 490 nm. Only Zn^{2+} and Cd^{2+} induced a large chelation enhanced fluorescence (CHEF) effect of **3** (4.4-fold enhancement for Zn^{2+} and 3.0-fold for Cd^{2+}). Thus, **3** can not distinguish between Zn^{2+} and Cd^{2+} , because they belong to the same group of the periodic table. However, only a much smaller amount of Cd^{2+} exists in the living system compared to Zn^{2+} , so we think that there is little influence on the imaging of Zn^{2+} . Transition metal ions (Cu^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+}) except for Mn^{2+} formed complex with **3**, and we observed a highly selective chelation enhanced fluorescence quenching (CHEQ) effect. Under alkali ions (Na^+ , K^+), alkaline earth ions (Ca^{2+} , Ba^{2+} , Mg^{2+}) and group III ion (Al^{3+}), the CHEF effect of **3** was not observed. These results suggested that only Zn^{2+} and Cd^{2+} induced CHEF effect of **3**.

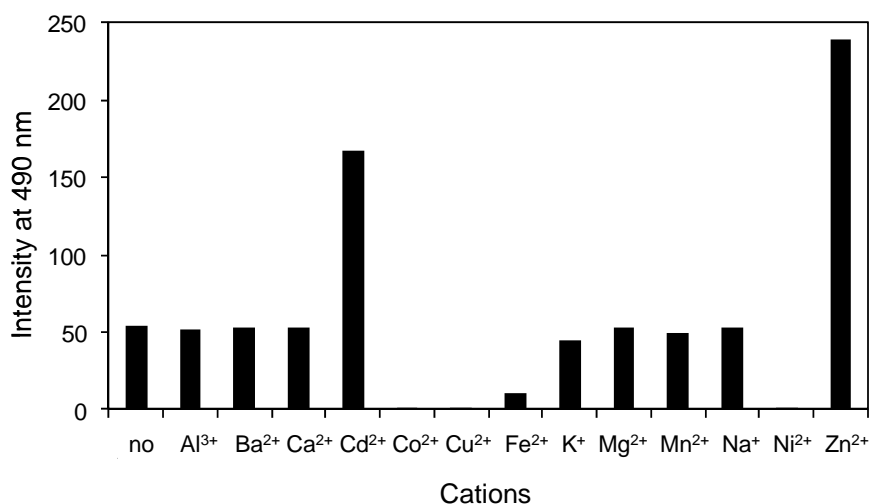


Fig. 5. Fluorescence intensity of **3** (1 μM) at 490 nm with addition of each metal cations (100 μM) in HEPES buffer (100 mM, 5% DMSO, pH = 7.4).

Although we could easily speculate that Zn^{2+} interacts with the bipyridyl moiety of **3**, we tried to understand the details of the binding mode by using the following experiments: ^1H NMR studies of **3** in the presence of Zn^{2+} and spectroscopic properties of the close analogs **4** and **6**. In ^1H NMR studies of **3** with zinc triflate, the NH / OH proton ($\delta = 11.09$ ppm) move to upfield shift ($\Delta\delta = 0.20$) and integral of the NH / OH proton does not change when the added Zn^{2+} was increase from 0 to 3 equiv. Protons at 3 and 3' position of pyridine rings move to downfield shift ($\Delta\delta = 0.63$ or 0.04 ppm) when 3.0 equiv. of zinc triflate was added (Fig. 6). The above spectral changes by addition of Zn^{2+} may indicate the complex formation between the bipyridyl moiety of **3** and Zn^{2+} . Effect of pH experiments also supported this result (Fig. 4). The fluorescence intensity with Zn^{2+} decreased in acidic conditions,

indicating that the protonation of the nitrogen atom of the pyridine ring may disturb the complex formation with Zn^{2+} . In addition we prepared the methoxy pyridine derivative **4** and the benzene analog **6** without the basic nitrogen atom at the end, both of which are closely related to **3** (Scheme 2). Analogs **4** and **6** lost the characteristic CHEF effects observed in the experiments of **3** with Zn^{2+} . No other metal cations did induce CHEF effects, however Fe^{2+} showed the strong CHEQ effects in both cases (Fig. 7). All together these results provided the further evidence that the interaction with Zn^{2+} takes place in the bipyridyl moiety of **3**. In addition the negative result with methoxy derivative **4** suggested that the NH or OH proton is essential in getting CHEF effects with Zn^{2+} . However at this point we are not able to conclude, which tautomer pyridine or pyridone of **3** is responsible for the chelation and CHEF effects with Zn^{2+} . Ajayaghosh et al. developed pyrrole end-capped 5,5'-divinyl-2,2'-bipyridyl derivative, a ratiometric fluorescence probe for Zn^{2+} which was designed to use the bipyridyl moiety as a metal chelator [33]. The pyridine-pyridone part of our newly developed **3** needs NH or OH proton for CHEF effects with Zn^{2+} . Thus it is likely that our Zn^{2+} fluorescent sensor **3** could be distinguished with previously reported bipyridyl examples.

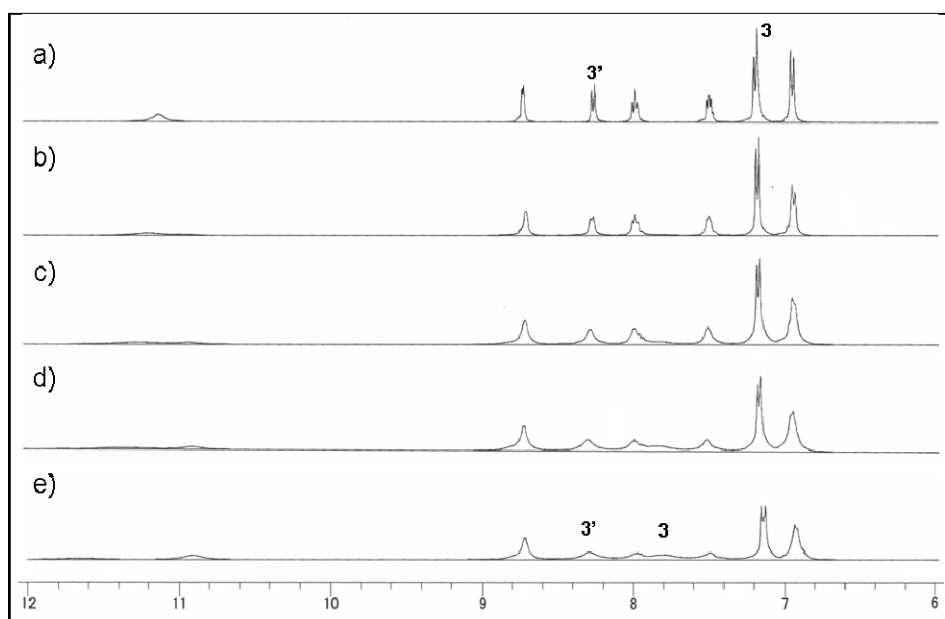
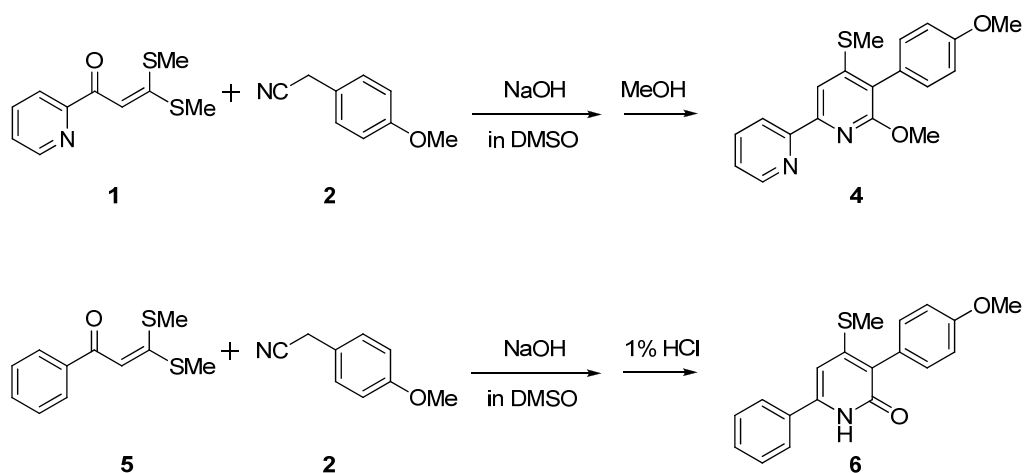


Fig. 6. ^1H NMR spectra in $\text{DMSO-}d_6$ upon addition of (a) compound **3**, (b) compound **3** + 0.5 equiv. Zn^{2+} , (c) compound **3** + 1.0 equiv. Zn^{2+} , (d) compound **3** + 2.0 equiv. Zn^{2+} , (e) compound **3** + 3.0 equiv. Zn^{2+} .



Scheme 2. Synthesis of compounds **4** and **6**.

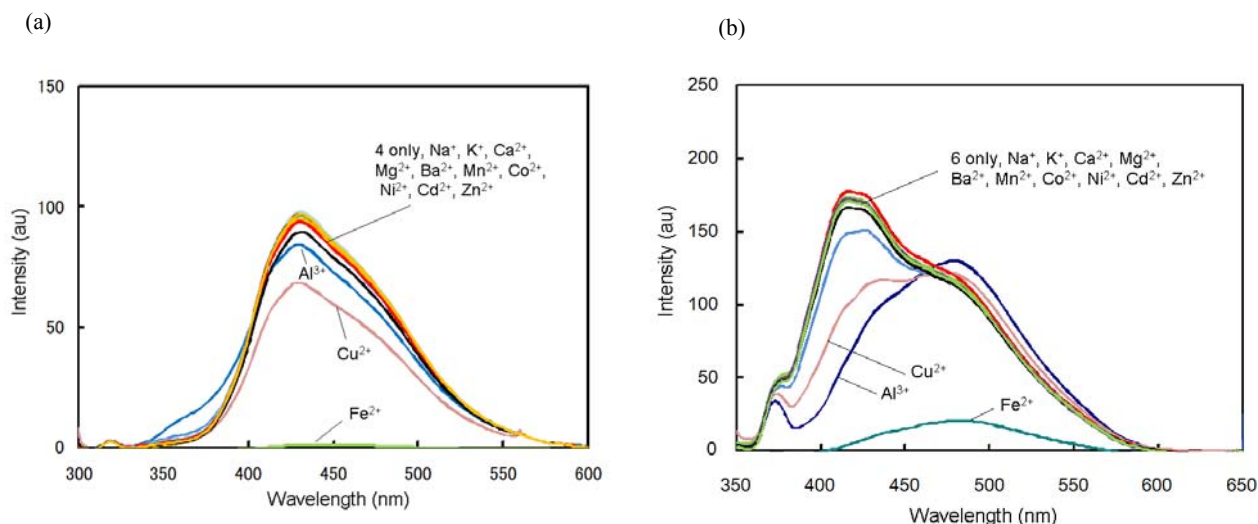


Fig. 7. Selectivity of **4** (a) and **6** (b) with addition of each metal cations (100 μM) in aqueous EtOH solution (1:1 EtOH/water (v/v)).

4. Conclusion

We found a novel and simple fluorophore, pyridine-pyridone derivative **3**. We believe that **3** would be a great starting point to design much improved Zn^{2+} selective fluorescent probes with desirable molecular characteristics for biological applications due to the following reasons: (1) The molecular

weight (MW = 324) is small enough. (2) Bipyridyl moiety as the chelating functionality for Zn²⁺ nicely shares with the fluorescent part, so the molecular weight can be kept minimal. (3) Despite its simplicity **3** already shows reasonable fluorescent properties and Zn²⁺ selectivity as a starting point. (4) Synthesis we used is simple, so this allows us to prepare a number of structurally related compounds. We are currently studying the next generation of bipyridyl based fluorescent probes related to **3** and will report on these results in due course.

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