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A low-molecular-weight fluorescent sensor with Zn^{2+} dependent bathochromic shift of emission wavelength and its imaging in living cells

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ABSTRACT

Novel, low-molecular-weight fluorescent sensors based on a pyridine–pyridone core structure, which functions as both the chelating part moiety for Zn^{2+} and as the fluorophore, were investigated. The length of the methylene spacer between the phenyl ring and the 3-position of the pyridone ring greatly influenced the emission wavelength and intensity upon Zn^{2+} binding. 5-Benzyl-4-(methylsulfanyl)-[2,2′-bipyridin]-6(1*H*)-one (**2**) (MW = 308) with a methylene spacer of $n = 1$ showed good water solubility, a 30 nm bathochromic shift in its emission wavelength and an 18-fold fluorescence enhancement in response to Zn^{2+}. In addition, fluorescence microscopy imaging showed that **2** could be used to detect Zn^{2+} in living cells.

1. Introduction

Zinc has a variety of essential physiological functions in living systems. For example, it influences gene expression, apoptosis, enzyme regulation, immune system response, and neurotransmission [1–6]. Generally, Zn^{2+} is tightly chelated by proteins and displays structural and catalytic functions [7]. Zn^{2+} also exists in a free or chelatable form, and is believed to play an important role in biological systems [8,9]. Although significant efforts to understand the role of Zn^{2+} in physiology and particularly in the field of neurochemistry have been put forth, the effects of free Zn^{2+} in terms of human health and disease remain largely unexplored.

As such, fluorescent sensors for metal ions have attracted significant attention as optical materials in biosensing due to their high sensitivity. A variety of fluorescent sensors for Zn^{2+} have been developed, and some have been utilized in the analysis of Zn^{2+} in biological samples and have provided useful information regarding zinc biology [10–12]. In order to monitor Zn^{2+} in living cells, fluorescent sensors should have sufficient solubility in water in addition to adequate cell permeability. Most fluorescent sensors for Zn^{2+} typically have large molecular weights, because they are composed of a fluorescent core and a Zn^{2+} binding moiety. Therefore, their aqueous solubility and cell permeability may be limited. In a previous study, we reported low-molecular-weight fluorescent sensors for Zn^{2+} based on a pyridine–pyridone core structure [13]. The core structure acted as the chelating functionality for Zn^{2+}, and simultaneously functioned as the fluorescent moiety. As a result, the pyridine–pyridone derivatives exhibited good water solubility. In addition, substitution of the core with electron donating or electron withdrawing groups greatly affected the internal charge transfer (ICT) state. Accordingly, we developed fluorescence ON/OFF switching Zn^{2+} sensors by introducing a *p*-carboxyphenyl group at the 3-position of the pyridone ring [14]. However, to further understand the biological functions of Zn^{2+}, fluorescent sensors with high sensitivity, high selectivity, and low background fluorescence, etc. are still required.

In this study, we report novel fluorescent sensors with methylene spacers of varying lengths between the aryl ring and the

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pyridine–pyridone core. The spacer length greatly affected the fluorescence, as some led to a Zn^{2+} dependent bathochromic shift of the emission spectrum in addition to enhancements in the fluorescence. This may be an advantage of this sensor as bathochromic shifts in emission spectra enable researchers to precisely detect Zn^{2+}. Here, we describe the synthesis and fluorescence of the novel low-molecular-weight Zn^{2+} fluorescent sensors, and evaluate the potential of 5-benzyl-4-(methylsulfanyl)-[2,2'-bipyridin]-6(1*H*)-one (**2**) in biological systems.

2. Experimental

2.1. Materials and instruments

All solvents were of analytic grade and were used as received. ^1H and ^{13}C NMR were measured on a JEOL-GX-400 (400 MHz) and a Varian Mercury-300 (300 MHz) and the chemical shifts were reported as ppm (in DMSO-d6 and $CDCl_3$). HRMS were measured on a JMS-T100LP mass spectrometer. Mass spectra (MS) were recorded on a JEOL-DX-303 mass spectrometer and a JMS-T100LP mass spectrometer. Elemental analyses were carried out on a Perkin–Elmer instrument. Fluorescence spectra were obtained on a Jasco FP-6200 spectrofluorometer. Infrared (IR) spectra were recorded in potassium bromide pellets on a Jasco 810.

2.2. Synthesis of 4-(methylsulfanyl)-3-phenyl-6-(pyridin-2-yl) pyridin-2(1H)-one (**1**) [14]

A mixture of 1.13 g (5.0 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone, 0.70 g (6.0 mmol) of 2-phenylacetonitrile, and 0.80 g (20.0 mmol) of sodium hydroxide in 50 mL of DMSO was stirred for 2 h at room temperature (r.t.). The reaction mixture was poured into 300 mL of ice water and neutralized with 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 1% hydrochloric acid solution was refluxed for 1 h. After evaporation, the residual solid was recrystallized from methanol to give **1** (0.68 g, 2.3 mmol, 46%) as pale yellow crystals. Mp 281–283 °C. IR (KBr, cm^{-1}): 3370, 1695, 1610, 1550, 1510, 1460, 1420, 1065. ^1H NMR ($CDCl_3$, 400 MHz) δ 2.46 (s, 3H), 6.85 (s, 1H), 7.34–7.50 (m, 6H), 7.83 (ddd, J = 1.7, 8.0, 8.0 Hz, 1H), 7.90 (d, J = 7.8 Hz, 1H), 8.68 (d, J = 3.9 Hz, 1H), 10.61 (brs, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.40, 99.81, 119.48, 124.62, 128.17, 128.42, 130.06, 134.67, 137.28, 139.80, 147.85, 149.42, 151.87, 160.13. MS m/z: 295 (M^+ + 1, 3), 294 (M^+, 13), 248 (20), 247 (100), 140 (8), 106 (10), 79 (8), 78 (43), 51 (7), 44 (6). Anal. Calcd for $C_{17}H_{14}N_2OS$: C, 69.36; H, 4.79; N, 9.52%. Found: C, 69.37; H, 4.29; N, 9.35%.

2.3. Synthesis of 5-benzyl-4-(methylsulfanyl)-[2,2'-bipyridin]-6(1H)-one (**2**)

Compound **2** (0.22 g, 0.7 mmol) was prepared in 29% yield from 0.56 g (2.5 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone and 0.39 g (3.0 mmol) of 3-phenylpropanenitrile in a manner similar to that described for the synthesis of **1**. An analytical sample was recrystallized from methanol to give pale yellow crystals. Mp 181–182 °C. ^1H NMR (DMSO-d6, 300 MHz) δ 2.57 (s, 3H), 3.90 (s, 2H), 5.15 (brs, 1H), 7.12 (s, 1H), 7.19–7.24 (m, 6H), 7.51 (dd, J = 4.8, 5.1 Hz, 1H), 7.99 (dd, J = 6.3, 8.1 Hz, 1H), 8.23 (d, J = 7.8 Hz, 1H), 8.70 (d, J = 3.6 Hz, 1H). ^{13}C NMR (DMSO-d6, 75 MHz) δ 16.10, 32.87, 93.09, 117.21, 121.93, 126.51, 126.59, 128.82, 129.61, 129.98, 131.88, 139.93, 141.03, 146.22, 149.55, 151.86, 156.25, 159.71. MS m/z: 309 [M + H^+]. HRMS calcd for $C_{18}H_{17}N_2OS$ [M + H^+]: 309.1062. Found: 309.1056.

2.4. Synthesis of 4-(methylsulfanyl)-5-phenethyl-[2,2'-bipyridin]-6(1H)-one (**3**)

Compound **3** (0.10 g, 0.3 mmol) was prepared in 13% yield from 0.56 g (2.5 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone and 0.44 g (3.0 mmol) of 4-phenylbutanenitrile in a manner similar to that described for the synthesis of **1**. An analytical sample was recrystallized from methanol to give pale yellow crystals. ^1H NMR (DMSO-d6, 300 MHz) δ: 1.74 (t, J = 9.6 Hz, 2H), 2.16 (t, J = 9.6 Hz, 2H), 2.52 (s, 3H), 5.30 (brs, 1H), 7.09 (s, 1H), 7.15–7.29 (m, 6H), 7.61 (dd, J = 4.8, 6.8 Hz, 1H), 7.79 (dd, J = 6.4, 10.4 Hz, 1H), 7.95 (dd, J = 7.0, 10.4, 1H), 8.21 (d, J = 10.4 Hz, 1H), 8.68 (d, J = 5.6 Hz, 1H). ^{13}C NMR (DMSO-d6, 75 MHz) δ: 14.80, 27.28, 33.72, 93.68, 119.76, 121.31, 124.52, 124.80, 126.52, 129.00, 130.10, 134.19, 139.10, 140.37, 147.40, 149.27, 152.56, 157.62, 162.83. MS (ESI) m/z: 323 [M + H^+], 345 [M + Na^+]. HRMS calcd for $C_{19}H_{19}N_2OS$ [M + H^+]: 323.1218. Found: 323.1214.

2.5. Spectral measurements

The compound stock solution (1×10^{-2} M) was prepared by dissolution in DMSO. Solutions of perchlorate salts of Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, and Al^{3+} were prepared by dissolution in distilled water. For the fluorescence analysis, test compounds (10^{-6} M) were measured in HEPES buffer (100 mM, 5% DMSO, pH = 7.4) upon addition of Zn^{2+}. The binding stoichiometry of **1**–**3** to Zn^{2+} was investigated via a Job's plot. The dissociation constant (K_d) in HEPES buffer was determined by plotting the fluorescence intensity versus the free Zn^{2+} concentration. The selectivity of **2** was investigated in HEPES buffer (100 mM, 5% DMSO, pH = 7.4). The measurements were carried out at 298 K. The fluorescence quantum yields were measured with respect to a quinine sulfate solution (Φ = 0.54) as the standard.

2.6. Fluorescence microscope images in cells

RAW 264 macrophage cells (Riken Cell Bank, Tsukuba, Japan) and HepG2 hepatocellular carcinoma cells (Riken Cell Bank, Tsukuba, Japan) were cultured at 37 °C in a humidified atmosphere with 5% CO_2. They were grown in Dulbecco's modified Eagle's medium (DMEM) that included 10% FBS and 1% penicillin. The cells were incubated with 1:1 Zn^{2+}/pyrithione (100 μM) in culture media for 15 min at 37 °C. After washing with phosphate-buffered saline (PBS), the treated cells were incubated with **2** (30 μM) in culture media for 30 min at 37 °C. The incubated cells were imaged by fluorescence microscopy (Nikon Eclipse Ti).

3. Results and discussion

We previously reported that the bipyridyl form of the pyridine–pyridone core structure interacts with Zn^{2+}, and that the NH/OH proton of the pyridone ring is essential for chelation-enhanced fluorescence (CHEF) effects upon binding Zn^{2+} [13]. In addition, different phenyl groups (phenyl, *p*-methoxyphenyl, *p*-chlorophenly etc.) at the 3-position of the pyridone ring affect the fluorescence intensity upon Zn^{2+} binding [14]. In order to understand the role of the phenyl ring, we prepared compounds **1**–**3** with methylene spacers (between the phenyl ring and the 3-position of the pyridone ring of the pyridine/pyridone core) of different lengths (**1**: n = 0, **2**: n = 1, **3**: n = 2). Compounds **1**–**3** were obtained via a simple one-pot reaction of 3,3-bis(methylsulfanyl-1-pyridin-2-yl)propenone with phenylacetonitriles in the presence of powdered sodium hydroxide in dimethyl sulfoxide (DMSO), followed by treatment with 1% HCl (Scheme 1).