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A high-affinity fluorescent Zn^{2+} sensor improved by the suppression of pyridine-pyridone tautomerism and its application in living cells

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A high-affinity and low-molecular-weight fluorescent Zn^{2+} sensor **1** based on a 2,2′-bipyridine scaffold, which functions as both the chelating moiety for Zn^{2+} and the fluorophore, was developed and evaluated in biological applications. Controlling the occurrence of tautomerism of the chelating moiety for Zn^{2+} by introducing an amino group at the 6-position of the pyridine ring dramatically increased the binding affinity toward Zn^{2+}. Fluorescent sensor **1** exhibited a nanomolar-range dissociation constant (K_d = 2.2 nM), a large Stokes shift (140 nm), and an 8.6-fold turn-on response to Zn^{2+} under physiological conditions. Fluorescence images revealed that fluorescent sensor **1** exhibits good properties with respect to aqueous solubility and cell permeability and can quantitatively detect the Zn^{2+} levels in living cells. Furthermore, a $^{65}Zn^{2+}$ radioactive zinc isotope uptake study revealed the real concentration of accumulated Zn^{2+} at the detection limit. The novel fluorescent sensor **1** is a promising sensor for use in biological applications.

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1. Introduction

Fluorescent sensors, which are based on fluorophores such as organic small molecules, fluorescent proteins, or quantum dots, are a powerful tool for visualizing proteins, peptides, and small molecules in biological processes [1,2]. Sensors designed for target identification, delivery, and optical properties such as fluorescence on-off control are required to visualize specific biological processes. Toward this end, various small organic molecules whose properties are relatively easy to optimize have been developed.

Metal ions in living organisms play critical roles in many biochemical processes [3]. The concentration levels of metal ions are accurately controlled. Therefore, detecting the changes in the concentration and distribution of various metal ions in living organisms by fluorescent sensors provides extensive information related to biological processes.

Zn^{2+} is also an essential trace element in living systems, and it plays important roles in many biochemical processes, including gene expression, apoptosis, enzyme regulation, immune system response, and neurotransmission [4–9]. Most Zn^{2+} ions are tightly bound to proteins to enable the formation of a proper three-dimensional (3D) structure and to create catalytic centers for enzymes. Zn^{2+} also exists in free or chelatable form in living cells, with concentrations in the subnanomolar-to-millimolar range [9–12]. The chelatable Zn^{2+} ions are involved in essential biological functions and mediate the pathophysiology of diseases [13–17]. Zn^{2+}-selective fluorescent sensors are valuable tools for investigating the roles of Zn^{2+} in living systems. A number of fluorescent sensors have recently been developed [18–27] and have greatly advanced our understanding of the role of Zn^{2+} in physiology, particularly in the field of neurochemistry. In principle, several criteria exist for a Zn^{2+}-selective fluorescent sensor that functions in biological samples. Selectivity toward Zn^{2+} over other components in the living system, including Na^+, K^+, Mg^{2+}, and Ca^{2+}, which exist in millimolar concentrations, is essential. A high affinity toward Zn^{2+} such that the dissociation constant (K_d) is similar to the existing Zn^{2+} concentration in the sample is also necessary. Other desirable features for the sensor include properties such as clear turn-on and

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turn-off functions and good aqueous solubility. However, no universal sensor that can be used in all Zn^{2+} imaging studies exists; in particular, the acquisition of quantitative fluorescence images in a cell is difficult. Hence, research toward the development of new Zn^{2+} sensors with improved physicochemical, chemical, and optical properties continues.

Recently, we developed low-molecular-weight (MW = ca. 300) fluorescent sensors for Zn^{2+} by using a pyridine–pyridone scaffold [28–30]. Despite their simplicity and low molecular weight, these pyridine–pyridone-based sensors exhibited Zn^{2+} selectivity (K_d = 7–30 μM) and reasonably good fluorescence properties, including aqueous solubility. However, a sensor with greater affinity toward Zn^{2+} is needed to analyze the Zn^{2+} functions in living cells. In pyridine–pyridone sensors, we reported that the presence of an NH or OH proton is essential to achieve chelation-enhanced fluorescence (CHEF) effects with Zn^{2+} [28]. The pyridone ring of the pyridine–pyridone structure tautomerizes to the pyridine ring (keto–enol tautomerism), and Zn^{2+} interacts with the bipyridyl moiety (Fig. 1). However, we speculated that the pyridone tautomer that does not participate in the coordination with Zn^{2+} is responsible for the low affinity toward Zn^{2+}. In the amino–imino tautomerism of 2-aminopyridine, the amine tautomer is generally more stable than the imino tautomer [31]. Thus, we expected that a lower K_d value would be obtained if we synthesized such a structure instead of the pyridone ring and controlled the occurrence of the imino tautomer that does not participate in the coordination with Zn^{2+}. Herein, we report results obtained using a novel Zn^{2+} sensor **1** with high affinity toward Zn^{2+} (K_d = 2.2 nM). In addition, we have evaluated its ability to detect Zn^{2+} levels in cellular systems.

2. Experimental

2.1. Materials and instruments

All the solvents were analytical grade and were used as received. Metal-ion solutions were prepared by dissolving metal salts in distilled water. ^{1}H NMR and ^{13}C NMR spectra were measured using a JEOL-GX-400 (400 MHz) and a Varian Mercury-300 (300 MHz) spectrometer, with chemical shifts reported in ppm (in DMSO-d_6). High-resolution mass spectrometry (HRMS) measurements were performed using a JMS-T100LP mass spectrometer. Mass spectra (MS) were recorded using JEOL-DX-303 and JMS-T100LP mass spectrometers. Microanalyses were performed using a Perkin-Elmer model 2002. Fluorescence spectra were recorded using a Jasco FP-6200 spectrofluorometer. Ultraviolet (UV) absorption spectra were measured using a Hitachi 323 spectrophotometer. Infrared (IR) spectra were recorded in potassium bromide pellets using a JASCO 810 spectrometer.

2.2. Synthesis of 4-(methylsulfanyl)-5-phenyl-2,2′-bipyridin-6-amine (*1*)

Powdered sodium hydroxide (0.40 g, 10.0 mmol) was added to a solution containing 1.13 g (5.0 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone [32,33] and 0.70 g (6.0 mmol) of 2-phenylacetonitrile in 50 mL of DMSO, and the resulting mixture was stirred for 2 h at room temperature. The reaction solution was poured into 300 mL of ice water and neutralized with a 10% HCl solution. The mixture was extracted three times with 100 mL of dichloromethane. The combined organic extracts were washed with 200 mL of water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue and 150 mL of 28% aqueous ammonia was refluxed for 2 h. After evaporation of ammonia and water, the residue was chromatographed on a silica gel column using toluene as an eluent to obtain 0.44 g (1.51 mmol, 30%) of **1** as colorless needles. Mp: 119 °C–120 °C. IR (KBr, cm^{-1}): 3440, 3270, 3160, 1620, 1535, 1415, 700. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.50 (s, 3H), 5.26 (s, 2H), 7.27 (d, *J* = 7.3 Hz, 2H), 7.41 (m, 2H), 7.50 (dd, *J* = 7.3, 7.8 Hz, 2H), 7.70 (s, 1H), 7.91 (dd, *J* = 7.8, 7.8 Hz, 1H), 8.30 (d, *J* = 7.8 Hz, 1H), 8.67 (d, *J* = 4.4 Hz, 1H). ^{13}C NMR (DMSO-d6, 100 MHz) δ 13.9, 104.8, 117.5, 120.4, 123.9, 128.4, 129.3, 129.9, 129.9, 135.3, 137.0, 149.0, 149.7, 152.4, 155.4, 155.8. MS m/z: 294 (M^+ + 1, 25), 293 (M^+, 100), 292 (76), 278 (22), 260 (16), 246 (21). Anal. Calcd for $C_{17}H_{15}N_3S$: C, 69.59; H, 5.15; N, 14.32%. Found: C, 69.33; H, 5.04; N, 14.48%. HRMS Calcd for $C_{17}H_{15}N_3S$: 293.0987. Found: 293.0962.

2.3. Synthesis of N,N-dimethyl-4-(methylsulfanyl)-5-phenyl-2,2′-bipyridin-6-amine (*2*)

Powdered sodium hydroxide (0.20 g, 5.0 mmol) was added to a solution containing 0.65 g (2.5 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone and 0.35 g (3.0 mmol) of 2-phenylacetonitrile in 20 mL of DMSO, and the resulting mixture was stirred for 2 h at room temperature. The reaction solution was poured into 300 mL of ice water and neutralized with a 10% HCl solution. The mixture was extracted three times with 100 mL of dichloromethane. The combined organic extracts were washed with 200 mL of water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue and 2 mL of 50% dimethylamine was refluxed for 2 h. After evaporation of ammonia and water, the residue was chromatographed on a silica gel column using toluene as an eluent to obtain 0.28 g (0.9 mmol, 35%) of **2** as yellow-green crystals. Mp: 146 °C–147 °C. IR (KBr, cm^{-1}): 2924, 2858, 1459, 1375, 1097, 978, 727. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.48 (s, 3H), 2.71 (s, 6H), 7.28 (m, 2H), 7.37 (d, *J* = 7.3 Hz, 1H), 7.44 (m, 3H), 7.79 (m, 1H), 7.87 (s, 1H), 8.45 (d, *J* = 7.8 Hz, 1H), 8.67 (d, *J* = 3.9 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 15.4, 41.6, 100.1, 119.8, 123.6, 124.9, 128.5, 128.7, 128.8, 130.3, 130.6, 137.6, 140.1, 148.1, 149.1, 149.7, 152.3, 160.4. MS (ESI) m/z: 322 (M^+ + 1). HRMS Calcd for $C_{19}H_{19}N_3S$: 321.1300. Found: 321.1391.

2.4. Synthesis of 4-(methylsulfanyl)-5-phenyl-6-(pyrrolidin-1-yl)-2,2′-bipyridine (*3*)

Powdered sodium hydroxide (0.40 g, 10.0 mmol) was added to a solution containing 1.13 g (5.0 mmol) of 3,3-bis-methylsulfanyl-1-pyridin-2-yl-propenone and 0.7 g (3.0 mmol) of 2-phenylacetonitrile in 20 mL of DMSO, and the resulting mixture was stirred for 2 h at room temperature. The reaction solution was poured into 300 mL of ice water and neutralized with a 10% HCl solution. The mixture was extracted three times with 100 mL of dichloromethane. The combined organic extracts were washed with 200 mL of water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. A mixture of the residue and 20 mL of pyrrolidine was refluxed for 2 h. After evaporation of ammonia and water, the residue was chromatographed on a silica gel column using toluene as an eluent to obtain 0.87 g (2.5 mmol, 50%) of **3** as colorless needles. Mp: 157 °C–158 °C. IR (KBr, cm^{-1}): 2957, 2920, 2850, 1570, 1530, 1430, 1415. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.73 (m, 4H), 2.47 (s, 3H), 3.16 (m, 4H), 7.25–7.31 (m, 1H), 7.34–7.56 (m, 5H), 7.74 (s, 1H), 7.80 (ddd, *J* = 2.0, 8.0, 8.2 Hz, 1H), 8.46 (d, *J* = 8.2 Hz, 1H), 8.65 (m, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 15.3, 25.8, 49.9, 104.8, 119.7, 121.4, 123.5, 127.8, 128.2, 128.7, 130.3, 131.5, 136.9, 138.5, 149.0, 151.7, 152.1, 155.8, 157.1. MS (EI) m/z: 348 (M^+ + 1, 28), 347 (M^+, 100), 346 (54), 319 (43), 318 (70), 304 (27), 292 (12), 264 (12), 263 (57), 231 (10), 153 (15), 152 (13), 97 (10), 85 (15), 83 (13), 78 (22), 71 (18), 71 (17), 69

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