ORGANIC LETTERS

2006
Vol. 8, No. 21
4895−4898

# Methylazacalix[4]pyridine: En Route to $Zn^{2+}$-Specific Fluoresence Sensors

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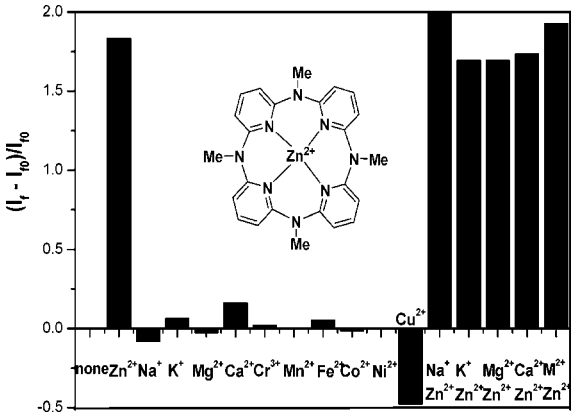
**Received August 3, 2006**

## ABSTRACT




**Owing to the electronic nature of the bridging nitrogen atoms which can adopt different hybridizations and form various conjugations with the adjacent pyridine(s), methylazacalix[4]pyridine underwent conformation and cavity preorganization to highly selectively bind the $Zn^{2+}$ ion. Rigidification and coplanarity of the macrocyclic ring led to a great enhancement of fluorescence of the intrinsic fluorescent host molecule.**

As the second most abundant heavy metal ion after iron in the human body, zinc ($Zn^{2+}$) plays important roles in various biological processes such as gene expression,[1] enzyme regulation,[2] neurotransmission,[3] and apoptosis.[4] Although $Zn^{2+}$ is generally bound to proteins and peptides,[5] a recent study in cell biology revealed a fraction of mobile $Zn^{2+}$ in some organs,[6] and the concentrations of chelatable $Zn^{2+}$ are estimated to range from ∼0.1 nM in the cytoplasm[7] to ∼0.1 mM in some vesicles.[8] To get deeper insight into the cytochemistry of the $Zn^{2+}$ ion, much effort has been devoted to measuring the concentration and the distribution of $Zn^{2+}$ ions in cells. As a consequence, a few zinc-selective fluorescent sensor molecules have emerged,[9−21] and some

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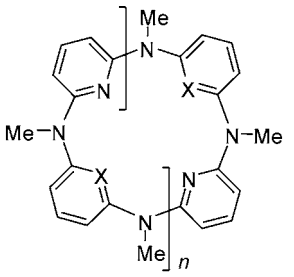
10.1021/ol061928k CCC: $33.50 
**Published on Web 09/21/2006**

of them have been shown to be suitable for the imaging of intracellular zinc.[14−16,18,19c−e,20,21]

Despite the rapid development of fluorescent detection of $Zn^{2+}$, molecular probes for specifically sensing $Zn^{2+}$ over other metal ions are still lacking.[18b] For example, the majority of the fluorescent molecules designed to date utilizes 8-arenesulfonamido-quinoline,[9−11,17a] bis(2-pyridylmethyl)-amine[13−15,18−20] and its analogues,[16] and aza-crown ethers[12,19b,22] as ligands to interact with the $Zn^{2+}$ ion. Almost all of the sensors, however, suffer competitive bindings from other transition-metal ions while they exhibit high binding affinity toward the $Zn^{2+}$ ion. In fact, a few metal cations, especially divalent transition-metal ions, have comparable or even superior binding abilities to the $Zn^{2+}$ ion.[23] It is therefore highly challenging to design a $Zn^{2+}$-specific ligand and a fluorescent sensor molecule.

Recently, we[24−26] and others[27] have devised N-bridged calixheteroarenes as a novel type of macrocyclic molecule. By introducing nitrogen atoms into the bridge positions of calix[*n*]pyridines [*n* = 4, 8] and calix[*m*]arene[*n*]pyridines (Figure 1), for example, we have demonstrated that the




**1** X = N, *n* = 1 **3** X = CH, *n* = 1
**2** X = N, *n* = 3 **4** X = CH, *n* = 3

**Figure 1.** Structures of methylazacalix[*n*]pyridines (X = N, *n* = 2, 4) and methylazacalix[*m*]arene[*n*]pyridines (X = CH, *m* = *n* = 2, 4).

macrocyclic conformations and cavities can be fine-tuned due to the bridging nitrogens which are able to adopt $sp^2$ and/or $sp^3$ electronic configurations with and/or without the formation of conjugation systems with their one and/or two adjacent pyridine rings.[26] It was envisioned that the resulting fine-tunable cavity derived from azacalixpyridines might be suitable to discriminate marginally different cation species. After screening the azacalixaromatics[24−26] in our laboratory, we have found indeed that methylazacalix[4]pyridine (MACP-4) **1** is a very promising $Zn^{2+}$-specific fluorescent host molecule.

Spectroscopic data of methylazacalixpyridines **1**−**4** (Figure 1), which were synthesized utilizing a fragment coupling strategy from simple aromatic dibromide and diamine derivatives,[26] are compiled in Supporting Information Table S1. Although the macrocycles **2**−**4** did not change their absorption and emission spectra upon titration with $Zn^{2+}$, MACP-4 gave the highly $Zn^{2+}$-dependent UV−visible (Supporting Information, Figure S1) and fluorescence spectra (Figure 2, top). For example, in the absence of $Zn^{2+}$,

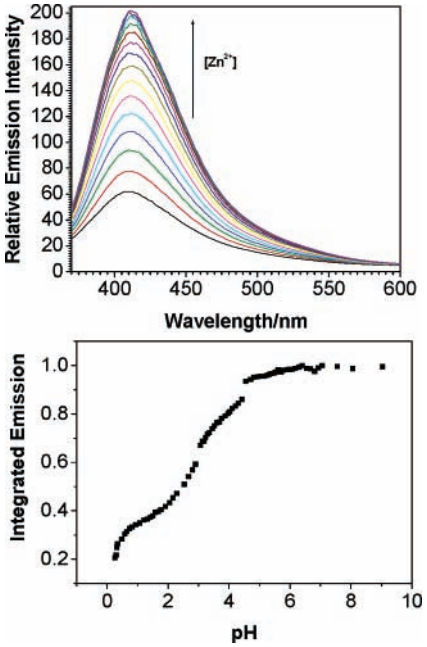



**Figure 2.** Fluorescence emission response of MACP-4 [18.7 $\mu$M in a mixture of acetonitrile and water (18:7, v/v)] to $Zn^{2+}$ solutions (0∼31.1 $\mu$M) (top) and fluorescence emission of the $Zn^{2+}$−MACP-4 complex at different pH values (bottom). The pH values were controlled by the addition of the solution of hydrochloric acid (12 M) in acetonitrile and the solution of aqueous $Me_4NOH$ (10%) in acetonitrile (18:7, v/v). Excitation was provided at 345 nm, and the excitation and emission bandwidths are 10 and 20 nm, respectively.

MACP-4 has an absorption maximum centered at 314 nm ($\epsilon$ = 2.09 × $10^4$ $M^{-1}$ $cm^{-1}$) with a corresponding weak emission maximum at 410 nm ($\Phi$ = 0.006). Upon the

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addition of up to 1 equiv of $Zn^{2+}$, three isobestic points at 234, 263.5, and 296.5 nm are observed in absorption spectra (Supporting Information, Figure S1), indicating an equilibrium between MACP-4 and its $Zn^{2+}$ complex. Very interestingly, the fluorescence intensity increases by about 3-fold ($\Phi = 0.010$) with no noticeable shift of the emission ($\lambda_{max} = 412$ nm) maximum (Figure 2). The Job's plot (Supporting Information, Figure S3) reveals the formation of a complex between MACP-4 and $Zn^{2+}$ with 1:1 stoichiometry. The binding affinity, which was calculated using the Hyperquad2003 program based on the emission responses at various $Zn^{2+}$ concentrations (Figure 2, top, and Supporting Information, Figure S2), is log $K = 5.97$, corresponding to a dissociation constant $K_d$ of 1.07 $\mu$M.

MACP-4 showed an extraordinary selectivity toward $Zn^{2+}$. As illustrated in Figure 3, MACP-4 (31.1 $\mu$M) did not respond to alkali and alkaline earth metal ions, and $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ did not change the $Zn^{2+}$-induced emission, even when present in large excess (1 mM). Except $Cu^{2+}$ which quenched the fluorescence of MACP-4, almost no influence of the first-row transition-metal ions such as $Cr^{3+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, and $Ni^{2+}$ (93.4 $\mu$M) on the fluorescence intensity of MACP-4 was observed. The presence of heavy metal ions including $Cd^{2+}$, a $d^{10}$ ion that often exhibits coordination properties similar to $Zn^{2+}$, $Hg^{2+}$, $Pb^{2+}$, and $Sn^{2+}$ (18.7 $\mu$M each), does not interfere with the emission of the $Zn^{2+}$−MACP-4 complex (Figure 3). To check the pH effect, the fluorescence of MACP-4 with saturating $Zn^{2+}$ under the different pH conditions was examined (Figure 2, bottom). It was found that fluorescence intensity remained almost constant above pH 5.0.

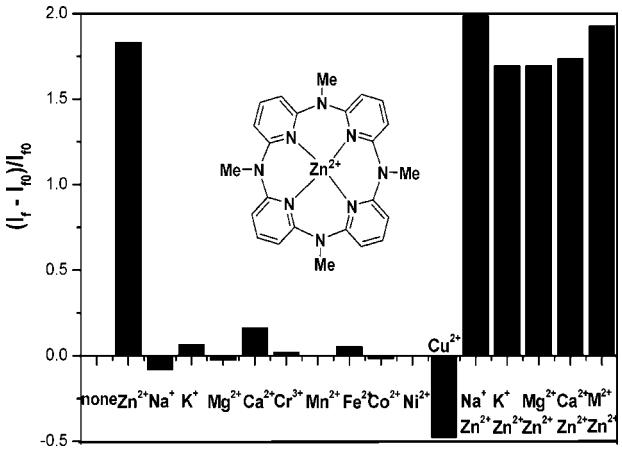


**Figure 3.** Emission intensity of MACP-4 in response to various metal ions. Concentrations: **1**, 18.7 $\mu$M; all alkali and alkali-earth metals, 1.0 mM; $Zn^{2+}$, 31.1 $\mu$M; $Cr^{3+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, 93.4 $\mu$M; $M^{2+}$ ($Cd^{2+}$, $Hg^{2+}$, $Pb^{2+}$, $Sn^{2+}$), 18.7 $\mu$M (each). Except $CrCl_3$, perchlorate ($ClO_4^-$) salts were used in all cases. Excitation was provided at 345 nm, and the emission was integrated between 370 and 600 nm.

To understand the intriguing fluorescence enhancement and quench of MACP-4 when binding $Zn^{2+}$ and $Cu^{2+}$, respectively, calculations of the energies of the molecular orbitals of MACP-4 and its complexes were performed at the theoretical level DFT/B3LYP/6-31G**. The initial conformations for the optimization of MACP-4 and its metal complexes were taken from their X-ray crystal molecular structures (Figure 4).[26,27] As depicted in Figure 5, the HOMO and LUMO of both MACP-4 and $Zn^{2+}$−MACP-4 are exclusively formed by the aromatic orbitals of the aminopyridine moiety, which determined the emission spectra at 410 nm. The increase of emission intensity on binding to $Zn^{2+}$ is therefore most likely the result of rigidification and coplanarity of the macrocyclic ring of the intrinsic fluorescent MACP-4. However, the d orbitals of $Cu^{2+}$ in the $Cu^{2+}$−

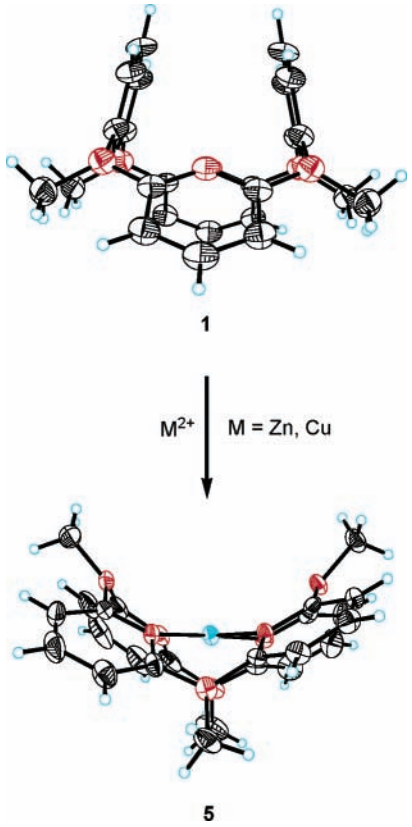


**Figure 4.** Formation of a complex **5** between an ion and MACP-4 **1**. The nitrogen atom is in red.

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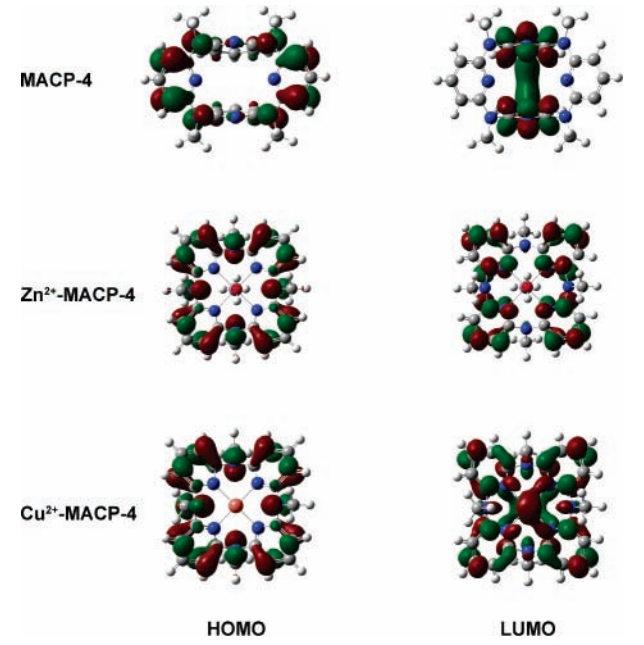


**Figure 5.** Theoretical calculation of HOMOs and LUMOs of MACP-4 **1** and its $Zn^{2+}$ and $Cu^{2+}$ complexes.

MACP-4 complex showed partial contribution to the LUMO, which led to the quench of the fluorescence of MACP-4 by $Cu^{2+}$ through the electron- and/or energy-transfer processes.

MACP-4, to the best of our knowledge, represents the most selective fluorescent ligand for the $Zn^{2+}$ ion. The outstanding selectivity of MACP-4 toward the $Zn^{2+}$ ion most likely originated from its unique fine-tuned cavity that is best fit for the $Zn^{2+}$ ion. As revealed by the X-ray crystal structures, to efficiently interact with a metal ion guest, MACP-4 has to change its 1,3-alternate conformation with $C_{2v}$ symmetry **1**[26] (Figure 4, top) into the saddle form with an approximate $D_{2d}$ symmetry **5** (Figure 4, bottom, and Supporting Information, Figure S5). All four bridging nitrogen atoms in MACP-4 adopt an $sp^2$ electronic configuration, and two of them form conjugative systems with one of the two edge-to-edge oriented pyridine rings.[26] In the complex, however, all four bridging nitrogens adopt a hybrid configuration between $sp^2$ and $sp^3$, and each nitrogen forms partial conjugation with both of its neighboring pyridine rings (Figure 4, top, and Supporting Information, Figure S5). It is the intrinsic nature of the bridging nitrogen atoms that enables conformation reorganization through the regulation of their electronic configuration and of the conjugation state with the adjacent pyridine rings. Because of the different size and different electronic nature, ions other than $Zn^{2+}$ are not efficiently interacted by the resulting cavity of the saddle-shaped MACP-4. It should be noted, however, that the $Cu^{2+}$ ion, which has the same ion radius as $Zn^{2+}$, also forms a complex **5** with MACP-4 (Figure 4, bottom, and Supporting Information, Figure S5). Because the $Cu^{2+}$ ion only induces a fluorescence quench of MACP-4, it is easy to differentiate $Zn^{2+}$ from $Cu^{2+}$. Besides, $Cu^{2+}$ exists at very low concentrations in biological samples, so it should have little effect on the application of MCAP-4 in sensing $Zn^{2+}$ in biological systems.[28]

In conclusion, we have shown that the macrocyclic ligand methylazacalix[4]pyridine (MACP-4) **1** is a highly $Zn^{2+}$-selective fluorescent sensing molecule. The selectivity stems from its unique conformation and cavity structure that are fine-tuned by the bridging nitrogens. The further modification of this intrinsic fluorescent macrocyclic ligand toward the improvement of the quantum yield, long excitation wavelength, and desired solubility would provide us with a useful $Zn^{2+}$-specific fluorescent probe.

**Acknowledgment.** This work was supported by the National Natural Science Foundation of China, the Chinese Academy of Sciences, and Ministry of Science and Technology in China.

**Supporting Information Available:** Spectroscopic data, UV−vis and fluorescence titration of **1** with $Zn^{2+}$ in the presence of other metal ions, and X-ray structure the of $Cu^{2+}$−MACP-4 complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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