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# Metal Ion Sensing Novel Calix[4]crown Fluoroionophore with a Two-Photon Absorption Property

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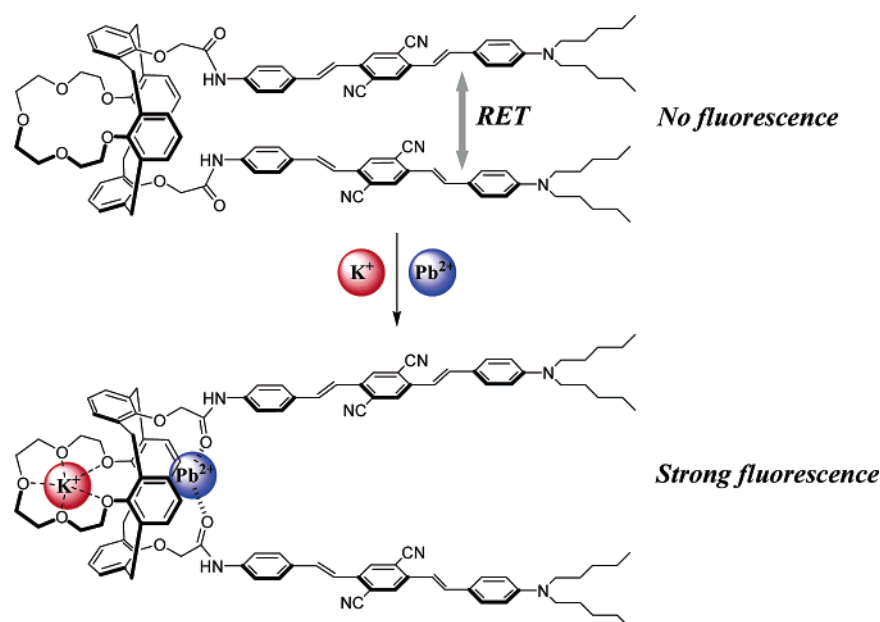
1,3-Alternate calix[4]arene-based fluorescent chemosensors bearing two-photon absorbing chromophores have been synthesized, and their sensing behaviors toward metal ions were investigated via absorption band shifts as well as one- and two-photon fluorescence changes. Free ligands absorb the light at 461 nm and weakly emit their fluorescence at 600 nm when excited by UV−vis radiation at 461 nm, but no two-photon excited fluorescence is emitted by excitation at 780 nm. Addition of an Al^{3+} or Pb^{2+} ion to a solution of the ligand causes a blue-shifted absorption and enhanced fluorescence due to a declined resonance energy transfer (RET) upon excitation by one- and two-photon processes. Addition of a Pb^{2+} ion to a solution of **1·K⁺** results in a higher fluorescence intensity than the original **1·Pb²⁺** complex regardless of one- or two-photon excitation, due to the allosteric effect induced by the complexation of K^+ with a crown loop.

Introduction

Fluorescent chemosensors capable of selectively recognizing cations have potential analytical applications in many different fields, including chemistry, biology, and medicine.[1−4] Most of the fluorescent chemosensors for cations are composed of a cation recognition unit (ionophore) together with a fluorogenic unit (fluorophore) and are called fluoroionophores.[3] An effective fluorescence chemosensor must convert the event of cation recognition by the ionophore into an easily monitored and highly sensitive light signal from the fluorophore.[5] Much attention has

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been paid to develop one-photon fluorescence (OPF) probes because they are useful tools for clarifying the functions in biological systems.[6] One of the major problems of such sensors is that the excitation wavelengths are in the range of 350−560 nm, which may cause damage to the substrates.[7] This problem could be avoided if one develops two photon sensors, which allow visualization of ions, small molecules, or enzyme activity in living cells by employing two-photon microscopy (TPM) that uses two NIR photons as the excitation source.[8−12] Additional advantages of TPM include improved penetration depth in cells and tissues as well as the reduced background emission due to the cellular autofluorescence.[13−15] For the maximum utilization of TPM, it is essential to develop efficient two-photon probes. However, there are only a few examples of two-photon sensors for metal ions, fluoride ions, and pH that have been studied in organic solvents or model membranes.[16,17] Therefore, the development of such probes is only beginning, and more fundamental knowledge is needed to facilitate this development.

Calixarenes with appropriate appended groups are good candidates as recognition moieties for cation probes because of their high selectivity toward specific cations.[18−26] A 1,3-alternate calix[4]crown platform provides a crown ether ring
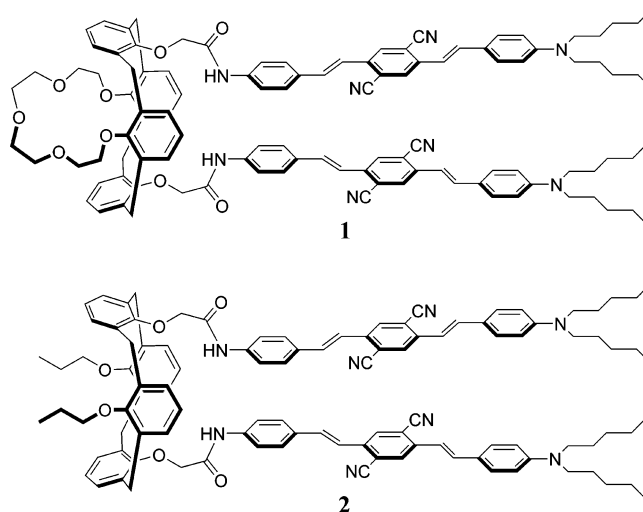

FIGURE 1. Structures of fluorescence chemosensors **1** and **2**.

for metal ion complexation with the potential for additional binding by cation−π interactions between the two rotated benzene rings and a polyether-complexed metal ion.[27] Reported calixarene-based fluorescence sensors utilize photophysical changes produced by cation binding: PET (photoinduced electron transfer);[21−24] PCT (photoinduced charge transfer);[19] excimer/exciplex formation and extinction;[28] or energy transfer.[29] However, in regards to selective sensing of specific metal ions, calix[4]arene-based TPF has never been exploited. So, we herewith report on the synthesis of PCT TPF appended calix-[4]arene molecules **1** and **2** (Figure 1) and their photophysical properties upon metal ion complexation. Furthermore, the two-photon absorption properties have also been studied during the binding events.

Results and Discussion

Our synthesis began with a preparation of two-photon absorbing molecule **3** as shown in Scheme 1. **5** was prepared by the reaction of **4** with 4-dipentylaminobenzaldehyde in the presence of NaOᵗBu in THF. Subsequently, reaction of **5** with 4-acetamidobenzaldehyde in the presence of NaOᵗBu followed by hydrolysis with HCl gave **3** in 37% yield.

Fluoroionophores **1** and **2** were prepared by the synthetic routes depicted in Scheme 2. The reaction of 25,27-bis-(chlorocarbonylmethoxy)calix[4]crown-5 (**6**)[21] and 25,27-bis-(chlorocarbonylmethoxy)-26,28-dipropyloxycalix[4]arene (**7**)[21] with **3** in the presence of triethylamine in THF provided **1** and **2** in moderate yields, respectively. Because the signaling units are conjugated with the metal ion recognition sites of facing amide groups, metal ion complexation is expected to cause the photophysical changes of the fluorophores such as wavelength

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SCHEME 1. Synthetic Route for Two-Photon Absorbing Fluorophore 3[a]
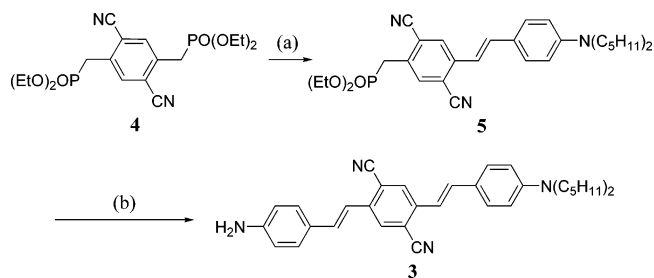
[a] Reagents and conditions: (a) 4-Dipentylaminobenzaldehyde/NaO′Bu/THF/5 h; (b) (i) 4-acetamidobenzaldehyde/NaO′Bu/THF/12 h, (ii) HCl/AcOH/120 °C /6 h.

SCHEME 2. Synthesis of Fluorescent Chemosensors 1 and 2
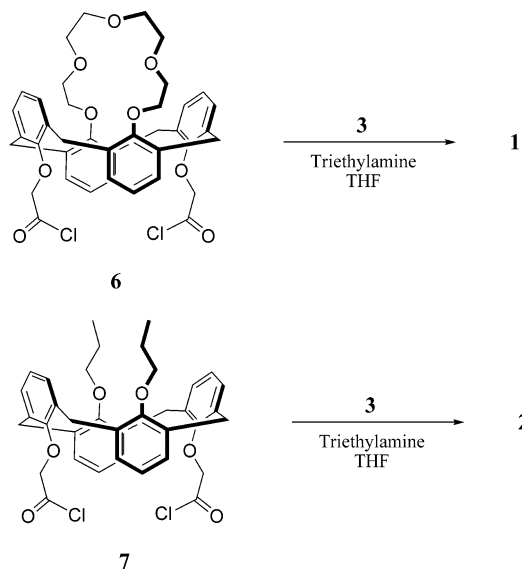
shifts and intensity changes in fluorescence spectra as well as in absorption spectra.

To obtain insight into the binding properties of **1** and **2** toward metal ions, we investigated the UV−vis spectrum changes upon addition of various metal perchlorates (Na$^+$, Li$^+$, K$^+$, Cs$^+$, Ca^{2+}, Mg^{2+}, Pb^{2+}, and Al^{3+}) to MeCN solutions of **1** and **2**, respectively. As shown in Figure 2, both **1** and **2** show a pronounced selectivity for Pb^{2+} and an even greater response

to the Al^{3+} ion with which the wavelengths of the ligands move from 460 to 385 nm whereas they scarcely change with other metal ions.

For the complexation ratio between the ligand and the metal ion, a Job plot experiment was conducted by varying the concentration of both **1** and Al^{3+} or Pb^{2+}. The maximum point at the mole fraction was 0.56 which refers to the typical ligand−metal complex ratio of 1:1 as shown in Supporting Information Figure S1.

To elucidate the blue shift induced by Al^{3+} ion binding to **1**, we investigated UV−vis spectrum changes of **1** upon addition of acid or base (Supporting Information, Figure S2). When HClO$_4$ was added to **1**, the peak at 460 nm disappeared probably because protonation on the *p*-dipentylamino group decreased the photoinduced charge transfer (PCT). On the other hand, addition of NaOH increased the long-wavelength band that can be attributed to enhanced PCT due to the extended conjugation by the deprotonation of the amide N−H bond. Therefore, the observed blue shift for the **1**·Al^{3+} complex indicates Al^{3+} ion binding to the two facing amide groups that decreased the PCT rather than to the crown loop of **1**. The similar blue shift observed in the **2**·Al^{3+} complex provides supporting evidence for this conclusion (Supporting Information, Figure S3).

Both **1** and **2** absorb the light at 385 nm and weakly emit their fluorescence at 520 nm as shown in Figure 3. The remarkably quenched emission of the free ligand is attributable to a resonance energy transfer (RET), so-called self-quenching, between the two facing fluorophores.[30] When an Al^{3+} ion is added to the CH$_3$CN solution of **1**, the Al^{3+} ion is encapsulated in the two facing amide groups to push the two facing fluorophores away from each other. This would decrease the RET and enhance the fluorescence emission. This view is in good agreement with above conclusion derived from UV−vis studies. Furthermore, no meaningful difference in the fluorescence emission is noted between **1** and **2** during the binding events, providing additional evidence that the crown loop of **1** does not participate in the binding of Al^{3+} and Pb^{2+} ions (vide supra).

It is well established that the crown ring of the calix[4]crown-5 can readily form the crown-5−K$^+$ complex. Therefore, there is a possibility that the formation of the crown-5−K$^+$ complex may cause an allosteric effect on the complexation of **1** with the Pb^{2+} or Al^{3+} ion.[31] When an excess amount of the K$^+$ ion was added to a solution containing **1**·Al^{3+}, there was no change in the fluorescence (Supporting Information, Figure S7). Simi-
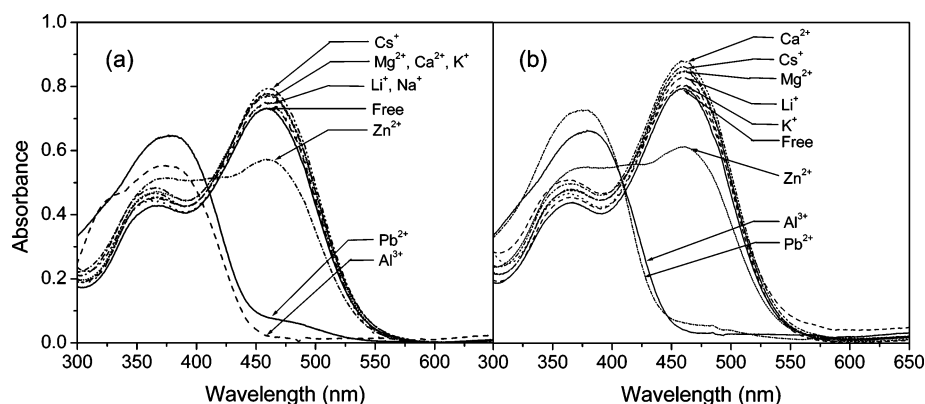

FIGURE 2. UV−vis spectra of (a) **1** and (b) **2** upon the addition of various metal ions in MeCN. Conditions: **1** and **2** (0.01mM)/CH$_3$CN; metal cations (500 equiv)/CH$_3$CN.
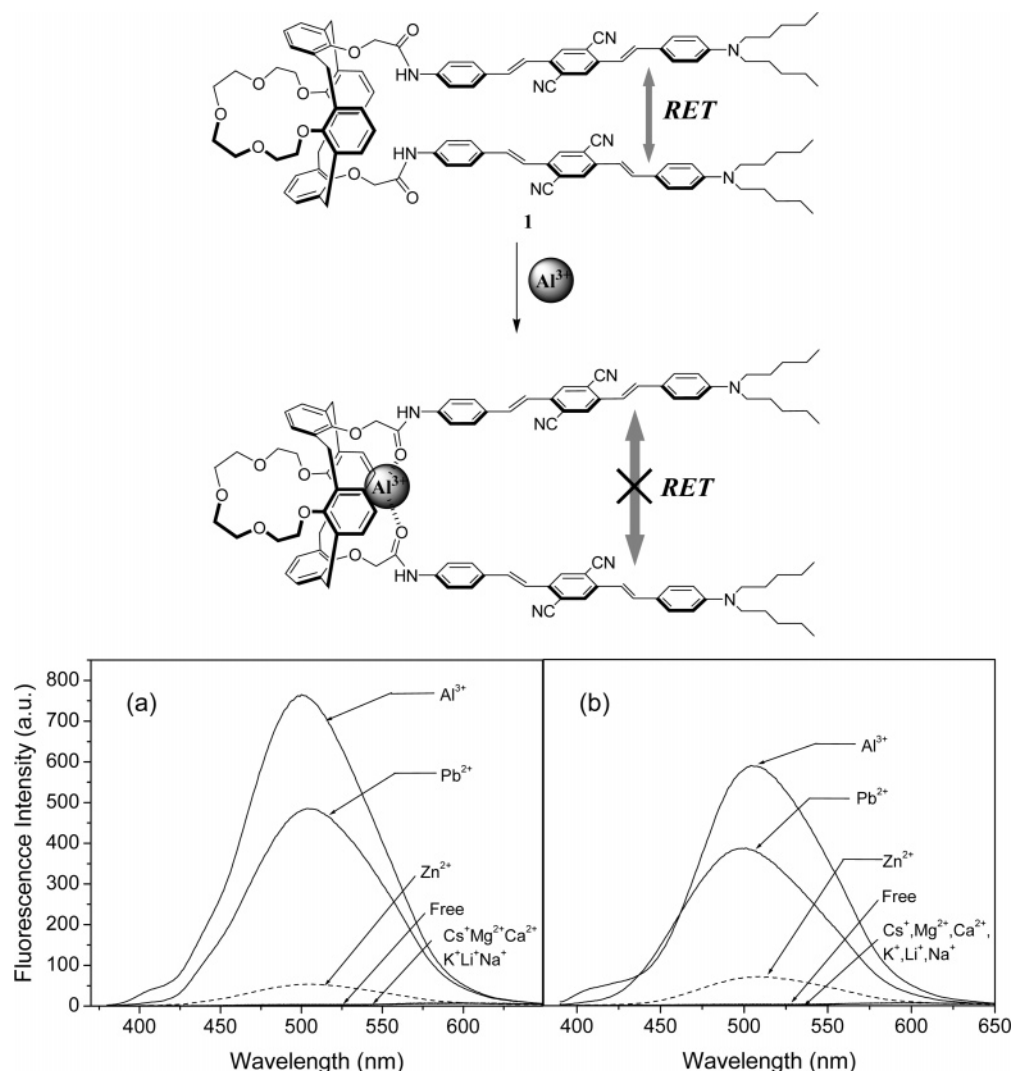
FIGURE 3. Fluorescence emission spectra of (a) **1** and (b) **2** upon the addition of various metal ions in MeCN. The excitation wavelength is 385 nm. Conditions: **1** and **2** (4.0 μM)/CH$_3$CN; metal cations (500 equiv)/CH$_3$CN.

TABLE 1. Photophysical Properties of 1 and 2

complex	$\lambda_{max}^{(1)}$ ^{a}	λ_{max}^{fl} ^{b}	Φ^c	$\lambda_{max}^{(2)}$ ^{d}	δ_{max}^e	$\Phi\delta_{max}$
1	460	520	0.017	NF	NF	NF
1 + Al^{3+}	385	500	0.27	780	571	154
1 + Pb^{2+}	385	500	0.085	780	996	85
1 + Pb^{2+} + K$^+$	385	500	0.10	780	998	100
2	460	520	0.019	NF	NF	NF
2 + Al^{3+}	385	500	0.16	780	655	105
2 + Pb^{2+}	385	500	0.078	780	872	68

^{a,b} λ_{max} of the one-photon absorption and emission spectra in nanometers. ^{c} Fluorescence quantum yield. ^{d} λ_{max} of the two-photon excitation spectra in nanometers. ^{e} The peak two-photon absorptivity in 10^{-50} cm^4 s/photon (GM). NF denotes no fluorescence.

larly, when the Al^{3+} ion was titrated into a solution of the **1·K$^+$** complex, we observed an enhanced fluorescence emission driven by metal ion exchange (Supporting Information, Figure S9).

More interestingly, when the K$^+$ ion was titrated into a solution of the **1·Pb^{2+}** complex, the fluorescence emission

increased as depicted in Figure 4. Moreover, addition of the Pb^{2+} ion to a solution of **1·K$^+$** gave an enhanced fluorescence emission and showed higher intensity than **1·Pb^{2+}**. The enhanced fluorescence emission can be attributed to an allosteric effect; that is, complexation of K$^+$ with the crown loop may have induced a conformational change that promoted the binding of the second metal ion (Pb^{2+}).

The two-photon cross sections (δ) of **1** and **2** were determined under various conditions by using the two-photon-induced fluorescence measurement technique.[32] Figure 5 shows the two-photon excitation spectra for the metal complexes of **1** and **2**. It was not possible to measure the δ values of **1** and **2** because the two-photon excited fluorescence (TPEF) was too weak to detect probably because of the RET. However, the metal complexes showed a significant two-photon cross section ranging from 385 to 998 GM (Table 1). The largest δ values are obtained for **1·Pb^{2+}** and **1·Pb^{2+}·K$^+$**. However, the two-photon action cross section ($\Phi\delta$) is largest for **1·Al^{3+}** because

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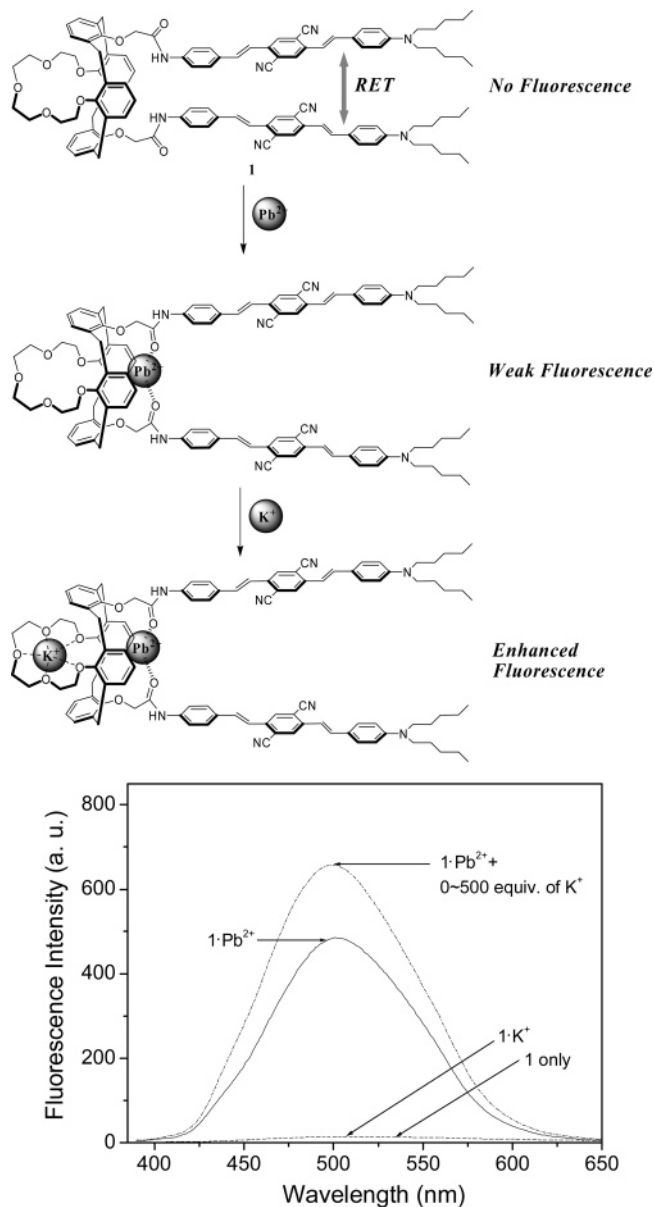
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FIGURE 4. Fluorescence changes of **1** (4.0 μM) with K$^+$ titration in the presence of Pb^{2+}. The excitation wavelength is 385 nm.

of the higher fluorescence quantum yield (Figure 6). All complexes show $\lambda^{(2)}_{max}$ at 780 nm. This is consistent with the prediction that the two-photon allowed state of a dipolar molecule should be close to the Franck−Condon state.[33]

As observed in the one-photon process, the TPEF intensity increased gradually upon addition of Al^{3+} and Pb^{2+} ions to **1** and **2**, respectively, and Al^{3+} showed a larger effect (Table 1). Moreover, the two-photon action cross section ($\Phi\delta$) is always larger for the Al^{3+} complex than for the Pb^{2+} complex. Furthermore, addition of K$^+$ to the solution of **1·Pb^{2+}** signifi-cantly enhanced $\Phi\delta$ and TPEF, indicating that the allosteric effect is also operating in the two-photon process (Table 1 and Figure 7). All of these results are in excellent agreement with those observed for the one-photon fluorescence studies. There-fore, it seems reasonable to conclude that similar mechanisms
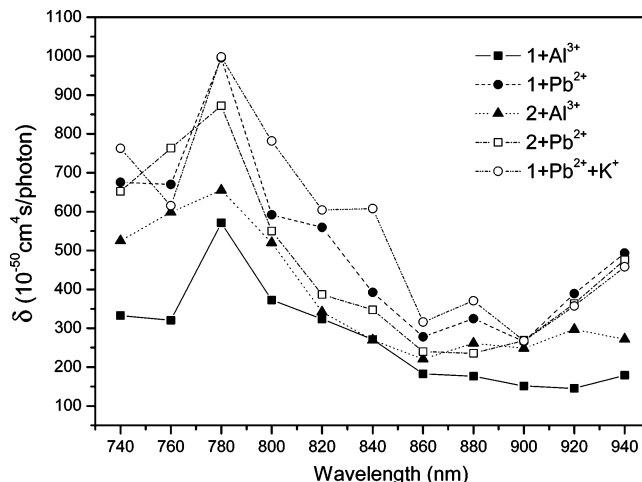
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FIGURE 5. Two-photon excitation spectra of **1** and **2** upon the addition of various metal ions in MeCN. Conditions: **1** and **2** (10 μM)/CH$_3$-CN; metal cations (500 equiv)/CH$_3$CN.

might be operating in the one- and two-photon processes during the binding events. This result provides additional support for the previous conclusion that efficient TPF probes for bioimaging applications could be designed by using the same strategy as that developed for the synthesis of one-photon fluorescent probes.[16]

In conclusion, we have synthesized 1,3-alternate calix[4]-arene-based fluorescent chemosensors (**1** and **2**) bearing two photon-absorbing chromophores and studied their sensing behaviors toward metal ions via absorption band shifts as well as one- and two-photon fluorescence changes. Addition of an Al^{3+} or Pb^{2+} ion to the ligands resulted in a blue-shifted absorption and enhanced fluorescence. Addition of a Pb^{2+} ion to a solution of **1·K$^+$** enhanced the fluorescence emission due to the allosteric effect induced by the complexation of K$^+$ with the crown loop. Identical results were observed for the two-photon processes, which can provide a useful design strategy for the synthesis of the two-photon sensors for biological applications.

Experimental Section

Synthesis. Compounds **4**,[34] **6**,[21a] and **7**[21a] were prepared following procedures reported in the literature.

25,27-Bis[*N*-(1-(2,5-dicyano-(1-(*p*-dipentylamino)styryl-4-styryl)-phenyl) aminocarbonyl)methoxy]calix[4]crown-5 in the 1,3-Alternate Conformation (1). A solution of **6** (0.30 g, 0.41 mmol), **3** (0.41 g, 0.82 mmol), and triethylamine (1.5 mL, 2.07 mmol) in 20 mL of dry THF was refluxed with stirring for 2 days and then evaporated in vacuo. The resulting solid was dissolved in CH$_2$Cl$_2$ (100 mL), and the organic layer was washed three times with water, dried over anhydrous MgSO$_4$, and evaporated in vacuo. The crude product was chromatographed on silica gel with EtOAc as an eluent to give 0.21 g (31%) of **1** as a reddish solid. Mp: 196−198 °C. IR (KBr pellet): 3298, 2232, 1678 cm^{-1}. FAB MS *m/z* (M$^+$) calcd 1667.9, found 1668.0. Anal. Calcd for C$_{108}$H$_{116}$N$_8$O$_9$: C, 77.76; H, 6.89. Found: C, 77.75; H, 6.87.

25,27-Bis[*N*-(1-(2,5-dicyano-(1-(*p*-dipentylamino)styryl-4-styryl)-phenyl)aminocarbonyl)methoxy]-26,28-dipropyloxycalix[4]-

(34) Wenseleers, W.; Stellacci, F.; Meyer-Friedrichsen, T.; Mangel, T.; Bauer, C. A.; Pond, S. J. K.; Marder, S. R.; Perry, J. W. *J. Phys. Chem. B* **2002**, *106*, 6853−6863.
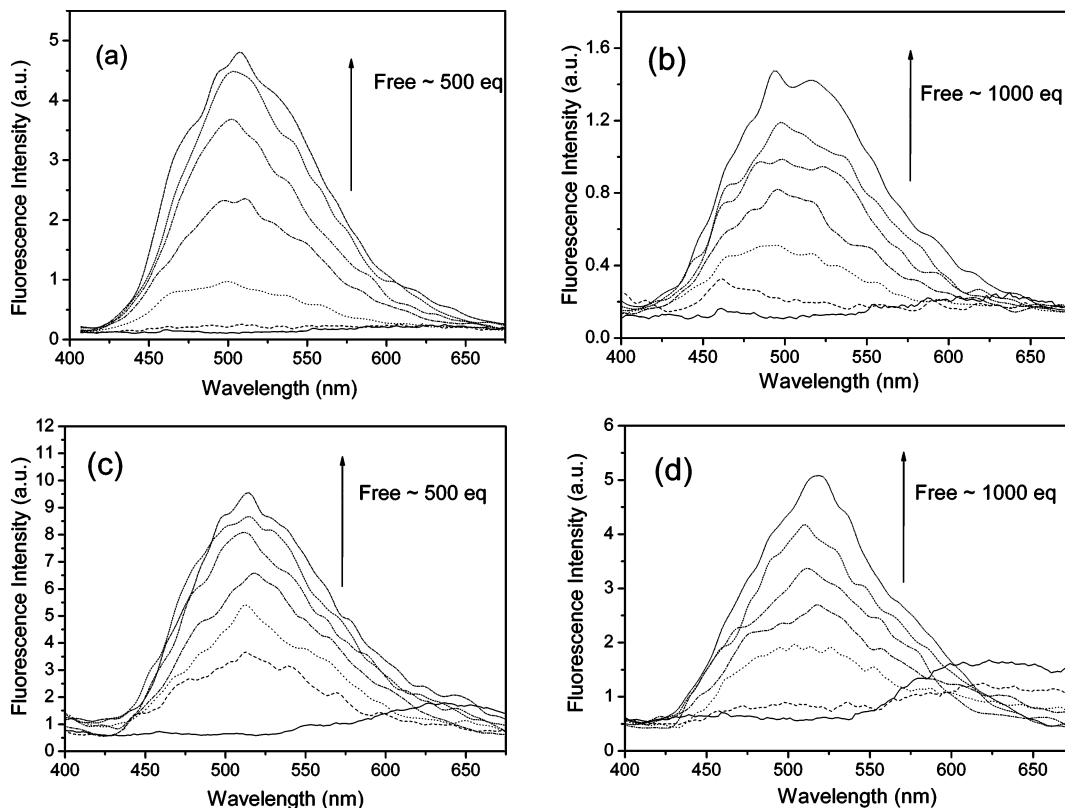
FIGURE 6. Two-photon excited fluorescence spectra of **1** (a, b) and **2** (c, d) in MeCN in the presence of 0, 0.5, 1.0, 5.0, 10, 100, and 500 equiv of Al^{3+} (a, c) and 0, 10, 100, 200, 300, 400, and 1000 equiv of Pb^{2+} (b, d). Conditions: **1** and **2** (4.0 μM)/CH$_3$CN. The excitation wavelength is 780 nm. The noise in the baseline at low metal ion concentration is due to the experimental errors associated with measuring weak signals.
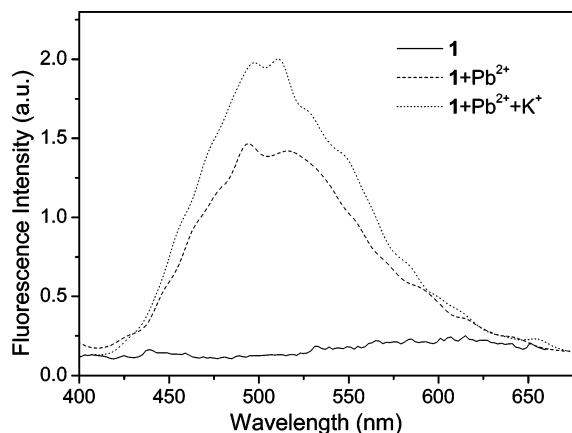

FIGURE 7. Two-photon fluorescence changes of **1** (4.0 μM) with K$^+$ titration in the presence of Pb^{2+}. The excitation wavelength is 780 nm.

arene in the 1,3-Alternate Conformation (2). It was prepared by modification of the procedure given above for **1**. The crude product was purified by column chromatography on silica gel with EtOAc–hexane (1:3) as eluent to give **2** in 37% yield. Mp: 172–174 °C. IR (KBr pellet): 3300, 2225, 1672 cm^{-1}. FAB MS *m/z* (M$^+$) calcd 1594.8, found 1595.1. Anal. Calcd for C$_{106}$H$_{114}$N$_8$O$_6$: C, 79.77; H, 7.15. Found: C, 79.75; H, 7.16.

2,5-Dicyano-[1-(*p*-dipentylamino)styryl-4-(*p*-amino)styryl]-benzene (3). NaO^{t}Bu (0.43 g, 4.5 mmol) was added dropwise to a solution of **5** (1.6 g, 3.0 mmol) in THF (40 mL) at 0 °C under N$_2$. To this solution was added dropwise 4-acetamidobenzaldehyde (0.54 g, 3.3 mmol), and the solution was stirred for 12 h at room

temperature. Water was added, and the product was extracted with EtOAc and washed with brine. The organic layer was dried over anhydrous MgSO$_4$, and the solvent was removed in vacuo to give 1.3 g (78%) of the crude product. Then, this intermediate was added to HCl/AcOH (2:1, 20 mL) at reflux for 6 h. A precipitate was formed upon cooling of the solution to room temperature. The product salt was filtered and washed with distilled water followed by recrystallization from CH$_2$Cl$_2$ and petroleum ether to give a red solid of **3**. Yield: 0.24 g (37%). Mp: 192–194 °C. IR (KBr, cm^{-1}): 3343, 1510 (NH$_2$), 2223 (CN). Anal. Calcd for C$_{34}$H$_{38}$N$_4$: C, 81.24; H, 7.62; N, 11.15. Found: C, 81.31; H, 7.59; N, 11.10.

1-(*p*-Dipentylamino)styryl-4-(diethoxyphosphoryl)methyl-2,5-dicyanobenzene (5). NaO^{t}Bu (1.0 g, 10 mmol) was added to a solution of **4** (3.0 g, 7.0 mmol) in THF (80 mL) at 0 °C under N$_2$. To this solution was added 4-dipentylaminobenzaldehyde (1.8 g, 6.9 mmol), and the solution was stirred for 5 h at room temperature. Water was added to quench the reaction, and the product was extracted with EtOAc and washed with brine. The organic layer was dried over anhydrous MgSO$_4$, and the solvent was removed in vacuo. Recrystallization with ethyl ether gave 1.6 g of **5** in 43% yield. Mp: 102–104 °C. Anal. Calcd for C$_{31}$H$_{42}$N$_3$: C, 69.51; H, 7.90; N, 7.84. Found: C, 69.57; H, 7.89; N, 7.88.

General Procedure for Fluorescence Studies. Fluorescence spectra were recorded with a RF-5301PC spectrofluorophotometer. Stock solutions (1.0 mM) of the metal perchlorate salts were prepared in MeCN. Stock solutions of free **1** and **2** (0.06 mM) were prepared in MeCN. For all measurements, excitation was at 385 nm with excitation and emission slit widths at 3 nm. Fluorescence titration experiments were performed using 4.0 μM solutions of **1** and **2** in MeCN and various concentrations of metal perchlorates

in MeCN. The fluorescence quantum yield was determined by using fluorescein Coumarin 307 as the reference by the literature method.[35]

Two-Photon Binding Studies and Measurement of a Two-Photon Cross Section. Two-photon fluorescence spectra were obtained with a DM IRE2 microscope (Leica) excited by a mode-locked titanium−sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at a wavelength of 780 nm and an output power of 1230 mW, which corresponds to approximately 12 mW of average power in the focal plane. Stock solutions (1.0 mM) of the metal perchlorate salts were prepared in MeCN. Stock solutions of free **1** and **2** (4.0 μM) were prepared in MeCN. The changes in the two-photon excited fluorescence intensity were measured upon addition of various amounts of metal ion concentration.

The two-photon cross section δ was determined by using a femtosecond (fs) fluorescence measurement technique as described.[32] **1** and **2** were dissolved in MeCN at concentrations of 1.0 × 10^{-5} M, and the two-photon induced fluorescence intensity was measured at 740−940 nm by using fluorescein (8.0 × 10^{-5} M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature.[36] The intensities of the two-photon induced fluorescence spectra of the reference and a sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated according to eq 1.

$$\delta = \frac{S_s \Phi_r \phi_r c_r}{S_r \Phi_s \phi_s c_s} \delta_r \qquad (1)$$

Supporting Information Available: Additional figures and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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