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A New Zinc(II) Fluorophore 2-(9-Anthrylmethylamino)ethyl-Appended 1,4,7,10-Tetraazacyclododecane

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A new 2-(9-anthrylmethylamino)ethyl-appended cyclen, L^3 (1-(2-(9-anthrylmethylamino)ethyl)-1,4,7,10-tetraazacyclododecane) (cyclen = 1,4,7,10-tetraazacyclododecane), was synthesized and characterized for a new Zn^{2+} chelation-enhanced fluorophore, in comparison with previously reported 9-anthrylmethylcyclen L^1 (1-(9-anthrylmethyl)-1,4,7,10-tetraazacyclododecane) and dansylamide cyclen L^2. L^3 showed protonation constants log K_{ai} of 10.57 ± 0.02, 9.10 ± 0.02, 7.15 ± 0.02, <2, and <2. The log K_{a3} value of 7.15 was assigned to the pendant 2-(9-anthrylmethylamino)-ethyl on the basis of the pH-dependent ^{1}H NMR and fluorescence spectroscopic measurements. The potentiometric pH titration study indicated extremely stable 1:1 Zn^{2+}–L^3 complexation with a stability constant log $K_s(ZnL^3)$ (where $K_s(ZnL^3) = [ZnL^3]/[Zn^{2+}][L^3]$ (M^{-1})) of 17.6 at 25 °C with $I = 0.1$ ($NaNO_3$), which is translated into the much smaller apparent dissociation constant K_d ($=[Zn^{2+}]_{free}[L^3]_{free}/[ZnL^3]$) of 2×10^{-11} M with respect to 5×10^{-8} M for L^1 at pH 7.4. The quantum yield ($\Phi = 0.14$) in the fluorescent emission of L^3 increased to $\Phi = 0.44$ upon complexation with zinc(II) ion at pH 7.4 (excitation at 368 nm). The fluorescence of 5 μM L^3 at pH 7.4 linearly increased with a 0.1–5 μM concentration of zinc(II). By comparison, the fluorescent emission of the free ligand L^1 decreased upon binding to Zn^{2+} (from $\Phi = 0.27$ to $\Phi = 0.19$) at pH 7.4 (excitation at 368 nm). The Zn^{2+} complexation with L^3 occurred more rapidly (the second-order rate constant k_2 is 4.6×10^2 M^{-1} s^{-1}) at pH 7.4 than that with L^1 ($k_2 = 5.6 \times 10$ M^{-1} s^{-1}) and L^2 ($k_2 = 1.4 \times 10^2$ M^{-1} s^{-1}). With an additionally inserted ethylamine in the pendant group, the macrocyclic ligand L^3 is a more effective and practical zinc(II) fluorophore than L^1.

Introduction

Development of new zinc(II) fluorescent sensors is attracting great interest.[1−14] Previously, the principle of PET (photoinduced electron transfer)-retarded fluorescence by Zn^{2+} has been reported for 1-(9-anthrylmethyl)-1,4,7,10-tetraazacyclododecane (**1**, L^1) and other macrocyclic homologues.[2] Despite an extremely strong Zn^{2+} uptake by the 1,4,7,10-tetraazacyclododecane ("cyclen") part (see **2**), its

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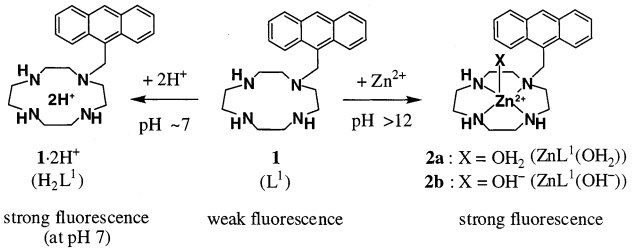
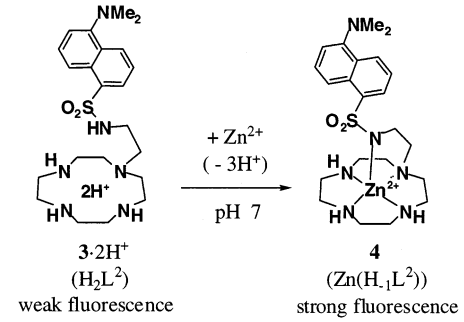
Scheme 1



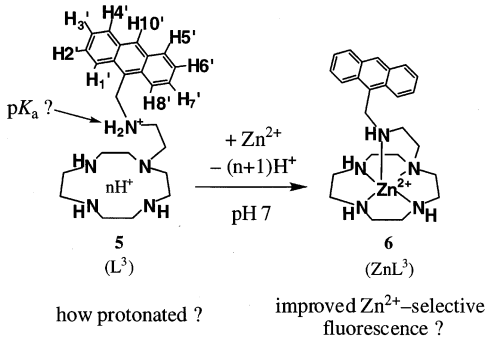
biggest drawback was a similarly occurring PET-retarded fluorescence by a competitive uptake of two protons at neutral pH (expressed as **1**·$2H^+$). Accordingly, to use L^1 as a Zn^{2+}-sensitive fluorophore, one must avoid the protonations; i.e., an extremely alkaline medium (pH > 12) should be employed (Scheme 1). Since then, however, the anthryl unit as a fluorescent group has been frequently incorporated in metal sensors.[1b,15]

On the other hand, we designed a dansylamide-pendant macrocycle such as **3** (L^2) as a new type of biomimetic Zn^{2+} chemosensor after fluorescent dansylamide binding to Zn^{2+} at the active center of carbonic anhydrase.[3,4] Indeed, L^2 strongly bound to Zn^{2+} in an amide-deprotonated complex, **4** ($Zn(H_{-1}L^2)$), at neutral pH, resulting in stronger dansylamide fluorescence from $\Phi = 0.03$ to $\Phi = 0.11$ (Scheme 2). The dissociation constant K_d of $Zn(H_{-1}L^2)$ was very small (8 pM) at pH 7.4, and therefore, the fluorescence with 5 μM L^2 at pH 7.3 was linearly responsive to a 0.1−5 μM concentration of Zn^{2+}, which was unperturbed by the presence of millimolar concentrations of other biological metal ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+}. Very recently, L^2 has been found to be cell-permeable and emit fluorescence upon binding to Zn^{2+} ions in HeLa cells.[16]

Scheme 2



Scheme 3



With new useful knowledge about competitive coordination vs protonation characters to pendant-attached cyclen,[17,18] we now have synthesized 2-(9-anthrylmethylamino)ethyl-pendant cyclen **5** (L^3) in our effort to develop new zinc(II) fluorophores. We were particularly interested in (1) the log K_a value of the new aminoethyl pendant, (2) whether PET is retarded by the protonation of this amine at neutral pH, (3) whether this potentially fifth donor amine binds to Zn^{2+}, and (4) how the PET-retarded fluorescence of L^1 by complexation with Zn^{2+} would be improved by the additionally inserted aminoethyl pendant group (Scheme 3). We have compared the complexation and fluorescent behaviors of L^3 with those of L^1 and L^2. Since detailed data with L^1 were not available,[2] we have simultaneously studied L^1 under the common conditions.

Experimental Section

General Information. L^1 was synthesized as described.[2] All other reagents and solvents used were of the highest commercial quality and used without further purification. $ZnCl_2$, $Fe(NO_3)_3 \cdot 9H_2O$, and $AgNO_3$ were purchased from Kanto Chemical Co. Ltd.

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$CdCl_2 \cdot 2.5H_2O$ and $Cd(NO_3)_2 \cdot 4H_2O$ were purchased from Wako Pure Chemical Industries Co. Ltd. $ZnSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 6H_2O$, $HgCl_2$, and $CuSO_4 \cdot 7H_2O$ were purchased from Yoneyama Yakuhin Kogyo Co. Ltd. $MnSO_4 \cdot H_2O$ and $Pd(NO_3)_2$ were purchased from Sigma-Aldrich Chemical Co., Inc. All aqueous solutions were prepared using deionized and redistilled water. Buffer (1 or 10 mM) solutions (KCl, pH 12.0; CAPS, pH 11.0, 10.0; CHES, pH 9.0, 8.5; HEPES, pH 8.0, 7.5, 7.4, 7.0; MES, pH 6.2, 6.0, 5.0; AcOH, pH 4.0) were used, and the ionic strengths of all were adjusted to 0.10 with $NaNO_3$. Good's buffer reagents (pK_a at 20 °C) were purchased from Dojindo and were used without further purification: CAPS (3-(cyclohexylamino)propanesulfonic acid; 10.4), CHES (2-(3-cyclohexylamino)-2-hydroxypropanesulfonic acid; 9.0), HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid; 7.6), and MES (2-morpholinoethanesulfonic acid; 6.2). Melting points were measured on a Yanaco melting point apparatus and are listed without correlation. IR spectra were recorded on a HORIBA FT-710 spectrometer. IR cards (type 62) were purchased from 3M Co. Ltd. ^{1}H NMR spectra were recorded on a JEOL JMN Lambda FT NMR spectrometer (500 MHz) or JEOL JMN Alpha FT NMR spectrometer (400 MHz). Tetramethylsilane (TMS) and 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid sodium salt (TSP) were used as internal references for ^{1}H NMR measurements in $CDCl_3$ and D_2O, respectively. The pD values in D_2O were corrected for a deuterium isotope effect using pD = pH-meter reading + 0.40. Elemental analysis was performed on a Perkin-Elmer CHN analyzer 2400. Thin-layer chromatography (TLC) and silica gel column chromatography were performed using Merck article 5554 (silica gel) TLC plates and Fuji Silysia Chemical FL-100D (silica gel), respectively.

1-Cyanomethyl-4,7,10-tris(*tert*-butylcarbonyl)-1,4,7,10-tetraazacycldodecane (8). 3Boc-cyclen (**7**)[19] (5.6 g, 12 mmol) was reacted with bromoacetonitrile (2.7 g, 23 mmol) in CH_3CN (40 mL) in the presence of Na_2CO_3 (1.3 g, 12 mmol) at 70 °C under an argon atmosphere for 2 days. After insoluble inorganic salts were removed, the filtrate was concentrated under reduced pressure. The remaining residue was purified by silica gel column chromatography (hexanes/AcOEt) to obtain **8** as a colorless amorphous solid (5.5 g, 91%). R_f = 0.6 (hexanes/AcOEt, 2:3). IR (KBr): 2231, 1678, 1458, 1363, 1252, 1171 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz, TMS): δ = 1.44 (s, 18H; C(CH_3)$_3$), 1.47 (s, 9H; C(CH_3)$_3$), 2.78−2.87 (br, 4H), 3.28−3.53 (m, 12H), 3.29−3.33 (br s, 2H). ^{13}C NMR ($CDCl_3$): δ = 28.52, 28.77, 46.50, 47.40, 49.93, 50.17, 53.98, 54.49, 79.62, 79.94, 80.25, 114.55, 155.15, 155.96, 156.12.

1-(2-(9-Anthrylmethylamino)ethyl)-1,4,7-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (9). A mixture of **8** (4.6 g, 9.0 mmol), Raney nickel (Aldrich (Raney 2800 nickel), 50% slurry in water), and 1 M NaOH (12 mL, 12 mmol) in EtOH (100 mL) was stirred under H_2 (20 atm) at room temperature for 5 days. After Raney nickel was filtered off with Celite (no. 545), the filtrate was evaporated. The remaining residue was purified by silica gel column chromatography (hexane/AcOEt and then MeOH) and then passed through aluminum oxide 90 (Merck, active basic (activity I), 70−230 mesh) to obtain 1-(2-aminoethyl)-4,7,10-tris(*tert*-butylcarbonyl)-1,4,7,10-tetraazacyclododecane[18b,c] as a colorless amorphous solid (3.1 g, 67% yield). R_f = 0.2 (MeOH). IR (IR card): 2974, 2931, 2810, 1687, 1462, 1414, 1365, 1250, 1169, 1153, 976, 773 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 1.45 (s, 9H; C(CH_3)$_3$), 1.47 (s, 18H; C(CH_3)$_3$), 2.55−2.88 (m, 4H; CH_2 of cyclen), 2.59 (t, J = 7.0 Hz, 2H; CH_2 of side chain), 2.83 (t, J = 7.0 Hz, 2H; CH_2 of side chain), 3.18−3.68 (m, 12H; CH_2 of cyclen). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.53, 28.69, 38.27, 48.10, 50.02, 54.67, 55.69, 56.70, 79.39, 155.44, 155.78, 156.22.

9-Chloromethylanthracene (920 mg, 4.1 mmol) was added to a mixture of 2-aminoethyl-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (2.1 g, 4.1 mmol) and Na_2CO_3 (860 mg, 8.1 mmol) in CH_3CN (150 mL) at 40 °C, and the whole was stirred for 12 h. Insoluble materials were filtered off, and the filtrate was concentrated under reduced pressure. The remaining residue was purified by silica gel column chromatography (eluent hexanes/AcOEt) to yield **9** as a pale yellow amorphous solid (1.7 g, 59%). R_f = 0.2 (hexanes/AcOEt, 1:5). IR (IR card): 2973, 2929, 2815, 1687, 1459, 1413, 1365, 1317, 1247, 1153, 1029, 975, 885, 860, 773, 755, 732 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 1.43 (s, 9H; (CH_3)$_3$), 1.47 (s, 18H; (CH_3)$_3$), 2.64−2.75 (m, 8H; CH_2), 3.01 (t, J = 6.9 Hz, 2H; CH_2), 3.15−3.52 (m, 12H; CH_2), 4.75 (s, 2H; NCH_2), 7.48 (dd, J = 8.0, 8.3 Hz, 2H; ArH(3′,6′)), 7.54 (ddd, J = 8.7, 7.9, 1.2 Hz, 2H; ArH(2′,7′)), 8.01 (d, J = 8.3 Hz, 2H; ArH (4′,5′)), 8.33 (d, J = 8.7 Hz, 2H; ArH(1′,8′)), 8.40 (s, 1H, ArH(10′) (for assignment, see Scheme 3). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.51, 28.72, 45.89, 46.07, 46.53, 47.71, 48.07, 49.96 53.84, 54.74, 56.07, 79.23, 79.48, 124.08, 124.97, 126.16, 127.30, 129.22, 130.29, 131.45, 131.57.

1-(2-(9-Anthrylmethylamino)ethyl)-1,4,7,10-tetraazacyclododecane Tetrahydrochloride Salt (5·4HCl·2H$_2$O). To a solution of **9** (1.6 g, 2.3 mmol) in EtOH (15 mL) was added 6 M aqueous HCl at room temperature. After being stirred for 5 h, the reaction mixture was concentrated under reduced pressure. The resulting crude powder was recrystallized from EtOH/H_2O to afford **5**·4HCl·2H_2O as a pale yellow powder (923 mg, 68%). R_f = 0.33 (MeOH/10% aqueous NaCl, 1:1). Mp: >250 °C. IR (KBr): 3409, 2958, 2769, 2439,2372, 1619, 1448, 1286, 1159, 1072, 960, 894, 846, 786, 736 cm^{-1}. ^{1}H NMR (500 MHz, D_2O, TSP): δ = 2.84−3.04 (m, 12H; CH_2), 3.08−3.23 (m, 8H; CH_2), 3.48 (t-like, 2H; CH_2-NHCH_2Ar), 5.36 (s, 2H; NCH_2Ar), 7.64 (dd, J = 7.7, 8.5 Hz, 2H; ArH (3′6′)), 7.76 (dd, J = 7.7, 8.7 Hz, 2H, ArH (2′7′)), 8.21 (d, J = 8.5 Hz, 2H; ArH(4′5′)), 8.33 (d, J = 8.7 Hz, 2H; ArH(1′8′)), 8.76 (s, 1H; ArH(10′)). ^{13}C NMR (100 MHz, D_2O): δ = 44.43, 44.90, 46.36, 47.44, 50.23, 50.67, 123.46, 125.56, 128.63, 130.89, 132.54, 133.35, 133.68, 133.89. Anal. Calcd for $C_{25}H_{43}N_5O_2Cl_4$: C, 51.11; H, 7.38; N, 11.92. Found: C, 50.78; H, 7.13; N, 11.68.

Potentiometric pH Titrations. The preparation of the test solutions and the calibration method of the electrode system (potentiometric automatic titrator AT-400 and auto piston buret APB-410 (Kyoto Electronics Manufacturing Co. Ltd.) with an Orion Research Ross combination pH electrode 8102BN) have been described previously.[3,18,19] All the test solutions (50 mL) were kept under an argon (>99.999% purity) atmosphere. The potentiometric pH titrations were carried out with I = 0.10 ($NaNO_3$) at 25.0 ± 0.1 °C using 0.1 M NaOH as a base, and at least two independent titrations were performed. Deprotonation constants and intrinsic complexation constants defined in the text were determined by means of the program BEST.[20] All the σ fit values defined in the program are smaller than 0.1. The K_W (=$a_{H^+}a_{OH}$), K'_W (=[H^+][OH^-]) and f_{H^+} values used at 25 °C are $10^{-14.00}$, $10^{-13.79}$, and 0.825. The corresponding mixed constants K_2 (=[HO^--bound species]a_{H^+}/[H_2O-bound species]) are derived using [H^+] = a_{H^+}/f_{H^+}. The species distribution values (%) against pH (=−log [H^+] + 0.084) were obtained using the program SPE.[20]

UV Spectrophotometric Titrations and Fluorescence Titrations. UV spectra and fluorescence emission spectra were recorded

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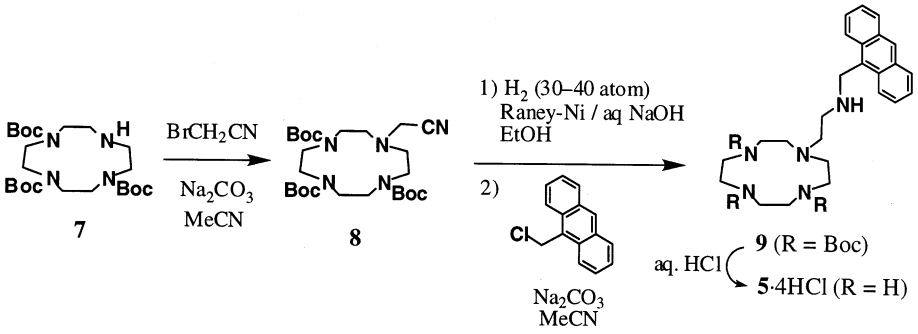
Scheme 4



on a Hitachi U-3500 spectrophotometer and a Hitachi F-4500 fluorescence spectrophotometer, respectively, at 25.0 ± 0.1 °C. For fluorescence titrations, a sample solution in a 10 mm quartz cuvette was excited at the isosbestic points determined by UV titrations. The obtained data of the fluorescence titrations (increases or decreases in fluorescence emission intensity at a given wavelength) were analyzed for apparent complexation constants K_{app} using the program Bind Works (Calorimetry Sciences Corp.). Quantum yields were determined by comparison of the integrated corrected emission spectrum of standard quinine, which was excited at 366 nm in 0.10 M H_2SO_4 (the quantum yield (Φ) is 0.55). Zinc(II) supplement capsules and tablets were purchased from FANCL Co. Ltd. (Japan) and Orihiro Co. Ltd. (Takasaki, Japan), and their sample solutions in water were prepared by dissolving zinc(II) powder contained in a capsule or a tablet in aqueous solutions.

Results and Discussion

Synthesis of 2-(9-Anthrylmethylamino)ethylcyclen (L^3, 5). The new ligand **5** was synthesized as summarized in Scheme 4. **7**[19] was allowed to react with bromoacetonitrile to obtain **8**. A nitrile group of **8** was reduced with H_2 in the presence of Raney nickel,[18b,c] and the resulting amino compound was treated with 9-chloromethylanthracene to obtain **9**. Deprotection of **9** with aqueous HCl yielded L^3 as a 4HCl salt.

Protonation Constants of 1 and 5. The protonation constants K_{ai} of **5** were determined by potentiometric pH titration of 1 mM L^3·5HCl (prepared from 1 mM L^3·4HCl·$2H_2O$ + 1 equiv of HCl) against 0.1 M NaOH with $I = 0.1$ ($NaNO_3$) at 25 °C (Figure 1). The titration data were analyzed for the acid−base equilibrium in eq 1, where a_{H^+} is the activity of H^+. The five protonation constants K_{ai} ($i = 1-5$) were calculated by using the program BEST.[20]

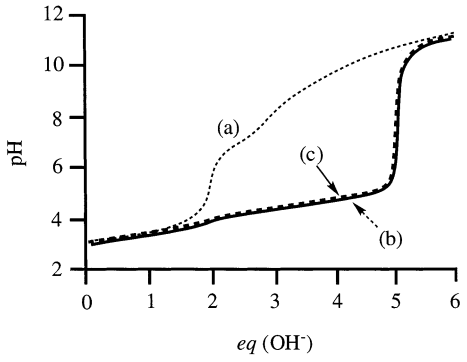


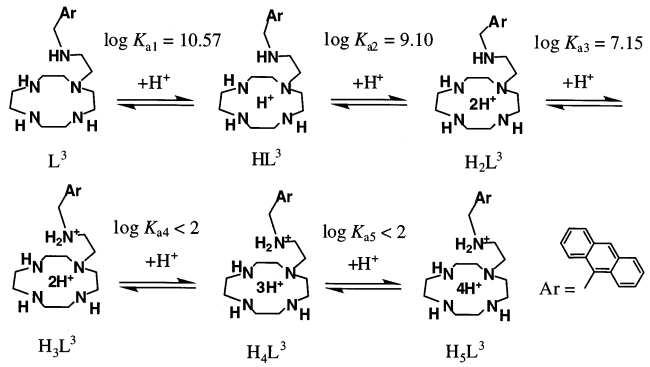
Figure 1. pH titration curves of 1 mM L^3·$5H^+$ (a), 1 mM L^3·$5H^+$ + 1 mM Zn^{2+} (b), and 1 mM L^3·$5H^+$ + 1 mM Cd^{2+} (c) at 25 °C with $I = 0.1$ ($NaNO_3$), where $eq(OH^-)$ is the number of equivalents of base added.

Table 1. Protonation Constants K_{ai} and Complexation Constants of Cyclen, 9-Anthrylmethyl-Pendant Cyclen **1** (L^1), Dansylamide-Pendant Cyclen **3** (L^2), and 2-(9-Anthrylmethylamino)ethyl-Pendant Cyclen **5** (L^3) at 25 °C with $I = 0.10$ ($NaNO_3$)[a]

	cyclen[b]	**1** (L^1)	**3** (L^2)[c]	**5** (L^3)
log K_{a1}	11.0	10.83 ± 0.03	11.8	10.57 ± 0.02
log K_{a2}	9.9	8.46 ± 0.03	10.8	9.10 ± 0.02
log K_{a3}	<2	<2	9.4	7.15 ± 0.02
log K_{a4}	<2	<2	4.0	<2
log K_{a5}			2.2	<2
log K_{a6}			<2	
log K_s(ZnL)	16.2	11.6 ± 0.1[e]	20.8	17.6 ± 0.1
log K_{app}(ZnL)[d]	10.6	7.3 ± 0.1	11.1	10.7 ± 0.1
log K_s(CdL)	14.3		19.1	17.5 ± 0.1

[a] For the definition of K_{ai}, K_s(ML), and K_{app}(ML), see the text. [b] From ref 21 at 25 °C with $I = 0.1$ ($NaNO_3$). [c] From ref 3a at 25 °C with $I = 0.1$ ($NaNO_3$). [d] Apparent complexation constants at pH 7.4 calculated from the potentiometric pH titration results. [e] The deprotonation constant pK_a for $ZnL^1(H_2O)$ (**2a**) ⇄ $ZnL^1(HO^-)$ (**2b**) + H^+ ($K_a = [\mathbf{2b}]a_{H^+}/[\mathbf{2a}]$) was 7.77 ± 0.03 at 25 °C with $I = 0.1$ ($NaNO_3$) (see Scheme 1).

Scheme 5



$$H_{i-1}L^3 + H^+ \rightleftarrows H_iL^3 \; (K_{ai} = [H_iL^3]/[H_{i-1}L^3]a_{H^+}) \; (i = 1-5) \quad (1)$$

Table 1 summarizes the log K_{ai} values for L^3 in comparison with those for L^1 and L^2. The four protonation constants log K_{a1}, log K_{a2}, log K_{a4}, and log K_{a5} for the cyclen nitrogens (Scheme 5) were nearly the same as the corresponding ones for the unsubstituted cyclen having log K_{ai} values of 11.0, 9.9, <2, and <2.[21] The remaining log K_{a3} value of 7.15 ± 0.02 for L^3 was assigned to the protonation of the amine in

(21) (a) Shionoya, M.; Ikeda, T.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1994**, *116*, 3848−3859. (b) Koike, T.; Kajitani, S.; Nakamura, I.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1995**, *117*, 1210−1219.

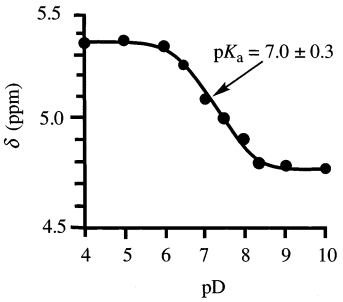


Figure 2. pH-dependent chemical shifts of ArCH_2N in the ^{1}H NMR titration of 2.0 mM L^3 in D_2O at 35 °C.

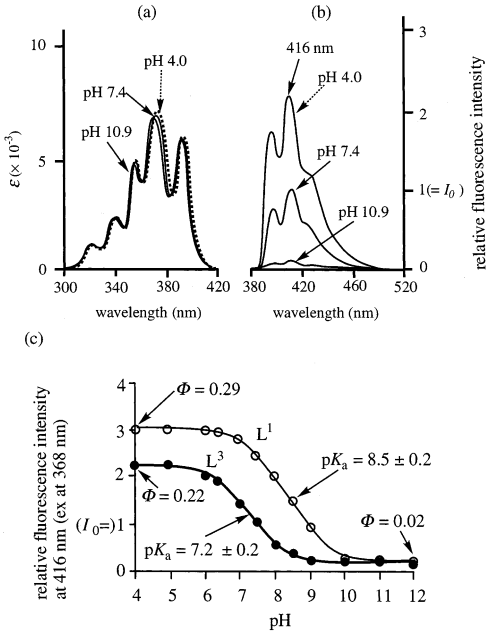


Figure 3. (a) UV spectra of L^3 (50 μM) in aqueous solution at pH 4.0 (10 mM acetate), 7.4 (10 mM HEPES), and 10.9 (10 mM CAPS) with I = 0.1 ($NaNO_3$) at 25 °C. (b) Fluorescence emissions spectra of L^3 (5 μM) at 25 °C with I = 0.1 ($NaNO_3$) (excitation at 368 nm) at pH 4.0 (1 mM acetate), 7.4 (1 mM HEPES), and 10.9 (1 mM CAPS) with I = 0.1 ($NaNO_3$) at 25 °C. (c) pH-dependent change of emission intensities of 5 μM L^1 (open circle) and 5 μM L^3 (closed circle) at 25 °C with I = 0.1 ($NaNO_3$). I_0 is the emission intensity of L^3 at pH 7.4 for Figure 3b and 3c.

the pendant, in collaboration with the results of ^{1}H NMR and fluorescence spectral titrations of L^3.

^{1}H NMR, UV Absorption, and Fluorescence Spectrophotometric pH Titration of L^3. To confirm the log K_{a3} value of 7.15 for the protonation of the pendant amine, the pH-dependent chemical shift of the anthrylmethylene protons of L^3 (2 mM) was studied in D_2O at 35 °C. The ArCH_2N singlet shifted from δ = 5.36 to δ = 4.79 as pD increased from 4.0 to 10.0, giving a sigmoidal curve as shown in Figure 2, from which the log K_{a3} value for L^3 of 7.0 ± 0.3 was obtained.

While the UV absorption spectra of L^3 (50 μM) changed little in the pH range 4.0−10.9 (Figure 3a), the fluorescence emission spectra significantly changed (Figure 3b). In acidic pH 4−5, where the pendant amine (log K_a = 7.15) is protonated, L^3 had a large fluorescence intensity (Φ = 0.22). As pH was raised, the pendant amine became less protonated and as a result the emission intensity decreased (Figure 3c). From this sigmoidal curve, the log K_a value of 7.2 ± 0.2 was calculated. The log K_a values for L^3 obtained by ^{1}H NMR (7.0 ± 0.3) and fluorescence titrations (7.2 ± 0.2) agreed with the log K_{a3} value of 7.15 determined by the potentiometric pH titrations. Likewise, for L^1 the log K_a value of 8.5 ± 0.2 (Figure 3c) agreed with 8.46 (Table 1) determined potentiometrically for the cyclen N bound to the anthrylmethyl side arm. PET from the anthrylmethylamine at pH 7.4 thus significantly occurred to L^3, but less so to L^1.[22] The weaker emission of L^3 than L^1 at neutral pH suggested that L^3 might be more responsive fluorometrically to Zn^{2+} complexation than L^1.

Study of Zinc(II) Complexation Properties of L^1 and L^3 by Potentiometric pH Titrations. The complexations of zinc(II) with L^1 and L^3 were determined by potentiometric pH titration of 1 mM L^1·4HCl and L^3·5HCl (from L^3·4HCl + 1 equiv of HCl) in the presence of an equimolar amount of $ZnSO_4$ at 25 °C with I = 0.10 ($NaNO_3$) (Figure 1b for L^3·5H^+ + Zn^{2+}). The 1:1 complexation constants K_s(ZnL) and the apparent 1:1 complexation constants K_{app}(ZnL) at pH 7.4, defined by eqs 2−4, were calculated and are summarized in Table 1. Evidently, ZnL^3 is much more stable than ZnL^1 due to the additional coordination of the amine in the pendant.

$$Zn^{2+} + L \rightleftarrows ZnL \qquad K_s(ZnL) = [ZnL]/[Zn^{2+}][L]\ (M^{-1}) \tag{2}$$

$$K_{app}(ZnL) = [ZnL]/[Zn^{2+}]_{free}[L]_{free}\ (M^{-1})\ \text{(at a given pH)} \tag{3}$$

$$[L]_{free} = \sum [H_nL]_{free}\ (n = 0-4\ \text{for}\ L^1,\ n = 0-5\ \text{for}\ L^3) \tag{4}$$

The pH-dependent speciation diagram (Figure 4a) for a mixture of 5 μM L^3 and 5 μM Zn^{2+} (the same concentrations for the fluorescence experiments) at 25 °C with I = 0.1 ($NaNO_3$) indicated nearly quantitative (>98%) complexation of L^3 with Zn^{2+} over pH 6.[23] Under the same conditions, L^1 (91%) was found to complex with Zn^{2+} (in the forms of 64% $ZnL^1(OH_2)$ (**2a**) and 27% $ZnL^1(OH^-)$ (**2b**)) at pH 7.4 (Figure 4b).[24] A similar Cd^{2+} complexation constant K_s(CdL) was determined (Figure 1c and Table 1).

For comparison, a speciation diagram for a mixture of 5 μM L^2 and 5 μM $ZnSO_4$ is shown in Figure 4c.[3] The quantitative (>99%) formation of **4** is evident at pH 7.4. However, compared with L^3, the complexation of L^2 with Zn^{2+} required more difficult deprotonation of the pendant sulfonamide (log K_a = 10.8; see Table 1) for its apical coordination. Accordingly, L^2 works as a zinc(II) sequestering agent at higher pH than L^3. The K_d value at pH 7.4, however, is 2.5 times smaller for ZnL^2 (K_d = $10^{-11.1}$ M)

(22) We presume that the lower log K_{a3} value (7.15) of L^3 may be due to a destabilization of the H_3L form (Scheme 5) by the repulsive interaction between the protonated side chain and the diprotonated cyclen ring.

(23) The FAB (fast atom bombardment) mass spectrum (positive) of ZnL^3 in aqueous solution (pH 8.0 ± 0.1) at m/z 468, 470, 472, and so on fits the theoretical distribution spectrum for $C_{25}H_{34}N_5Zn$ (($ZnL^3-H)^+$) (see the Supporting Information).

(24) The deprotonation constant pK_a for $ZnL^1(H_2O)$ (**2a**) ⇄ $ZnL^1(HO^-)$ (**2b**) + H^+ (K_a = [**2b**]a_{H^+}/[**2a**]) was determined to be 7.77 ± 0.03 by potentiometric pH titration at 25 °C with I = 0.1 ($NaNO_3$).

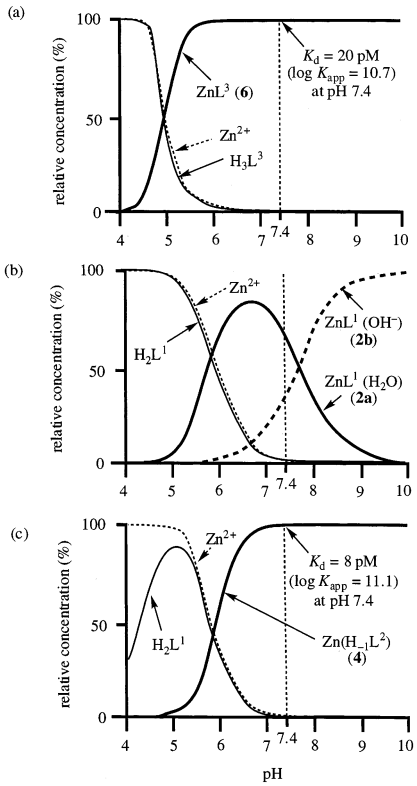


Figure 4. Speciation diagrams for 5 μM L^3 + 5 μM Zn^{2+} (a), 5 μM L^1 + 5 μM Zn^{2+} (b), and 5 μM L^2 + 5 μM Zn^{II} (c) as a function of pH at 25 °C with I = 0.1 ($NaNO_3$). For the structures of **2a** and **2b** in Figure 4a, see Scheme 1. In Figure 4c, species of less than 10% relative concentration were omitted for clarity.

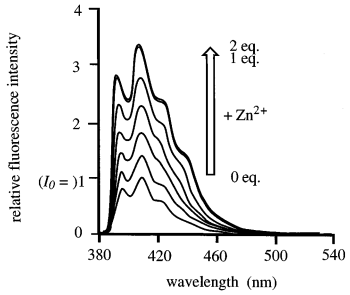


Figure 5. Spectral change in the fluorescence emission of 5 μM L^3 upon addition of Zn^{2+} (0−2 equiv) at pH 7.4 (1 mM HEPES with I = 0.1 ($NaNO_3$)) and 25 °C (excitation at 368 nm).

than that for ZnL^3 ($K_d = 10^{-10.7}$ M), since the deprotonated dansylamide anion apically binds to Zn^{2+} more strongly in **4** than the neutral amine in **6**.

Fluorometric Titrations of L^3, L^1, and L^2 with Zn^{2+}. The interaction of L^3 (5 μM) with Zn^{2+} (0−2 equiv) was examined by fluorometric signaling at pH 7.4 (1 mM HEPES with I = 0.1 ($NaNO_3$)) and 25 °C to see how the Zn^{2+} coordination would affect the PET from the anthylmethylamine. From the potentiometric titration results for L^3, we knew that almost 50% protonated amine pendant at pH 7.4 would become almost 100% Zn^{2+}-bound. As anticipated, Figure 5 displays the increasing emission of L^3 until an equivalent [Zn^{2+}]. The fluorescence titration curves of L^3, L^1, and L^2 with $ZnSO_4$ at common pH 7.4 are compared in Figure 6. The emission of L^3 increased about 3.1 times with an increasing [Zn^{2+}] (Φ = 0.14 → 0.44). The emission

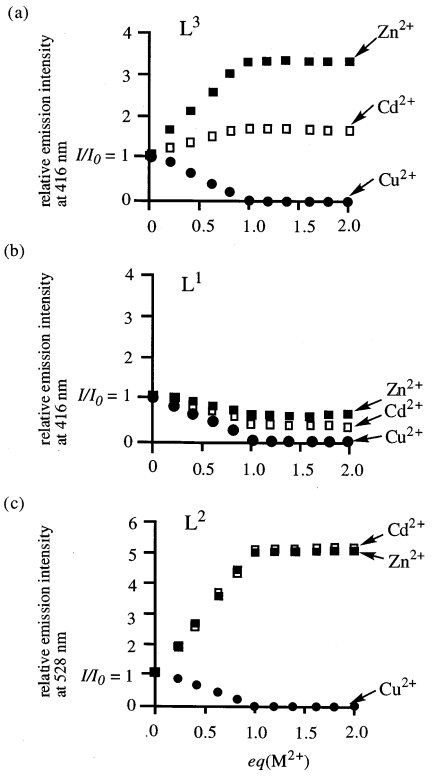


Figure 6. Fluorescence titration curves of L^3 (a), L^1 (b), and L^2 (c) at pH 7.4 (1 mM HEPES with I = 0.1 ($NaNO_3$)) and 25 °C with Zn^{2+} (closed squares), Cd^{2+} (open squares), and Cu^{2+} (open circles) ([L] = 5 μM) (excitation at 368 nm and emission at 416 nm for L^1 and L^3 and excitation at 330 nm and emission at 528 nm for L^2). $eq(M^{2+})$ is the number of equivalents of metal added against the ligands. I_0 values are the emission intensities of each ligand (5 μM) at 416 nm (for L^3 and L^1) or 528 nm (for L^2) in the absence of metal ions at pH 7.4.

increase in the case of L^3 is accounted for by the Zn^{2+} coordination of the anthrylmethylamine, whereby its PET is retarded. On the contrary, the emission of L^1 did not increase, but rather decreased a little (Φ = 0.27 → 0.19). In this case, the retardation of PET from the anthrylmethylamine would not change much by protonation or by Zn^{2+} coordination. A significant improvement of the Zn^{2+} signaling thus has been achieved by the insertion of an aminoethyl adjacent to the anthrylmethyl pendant.[25]

The fluorescent responses of L^2 to Zn^{2+}, Cd^{2+}, and Cu^{2+} under the same conditions are compared in Figure 6c (excitation at 330 nm and emission at 528 nm).[3] The detection limit of L^3 (5 μM) at pH 7.4 is [Zn^{2+}] = 0.1 μM,[26] which is almost the same as the reported limit for L^2 at the same pH. Despite the similar K_d(ZnL) and K_d(CdL) values, L^3 signaled Zn^{2+} more strongly than Cd^{2+} (Figure 6a,b), whereas L^2 unselectively responded to Zn^{2+} and Cd^{2+} (Figure

(25) From the fluorometric titration curves in Figure 6a,b, both apparent complexation constants log K_{app}(ZnL) for L^3 and L^1 in pH 7.4 buffer were estimated to be >7.

(26) To demonstrate a practical application of L^3, we analyzed the content of Zn^{2+} in capsules and tablets of commercially available zinc(II) supplements (purchased from FANCL Co. Ltd. and Orihiro Co. Ltd.) that contain various kinds of potentially disturbing ingredients. From an increase of emission of L^3 (5 μM) after addition of an aliquot of an aqueous solution of Zn^{2+} supplements, the Zn^{2+} contents were determined to be 7.5 ± 0.2 mg/capsule and 2.0 ± 0.3 mg/tablet, values agreeing with the indicated values (7.5 mg/capsule and 2 mg/tablet, respectively).

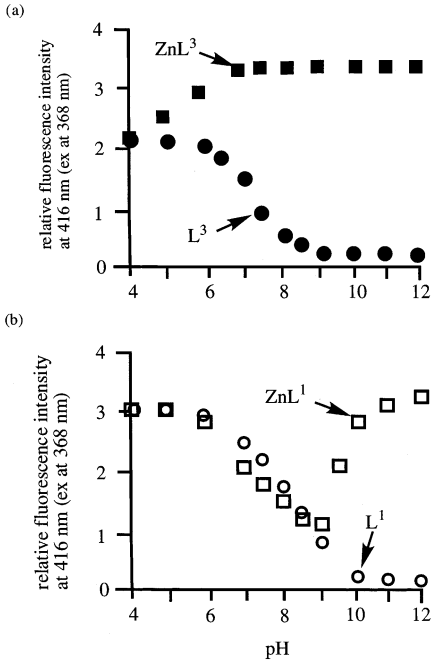


Figure 7. pH-dependent change of the fluorescence emission of L^3 (closed circles) and ZnL^3 (closed squares) (a) and L^1 (open circles) and ZnL^1 (open squares) (b) at 7.4 (1 mM HEPES with $I = 0.1$ ($NaNO_3$)) and 25 °C ([L] = [ZnL (prepared in situ)] = 5 μM). I_0 is the emission intensity at 416 nm of L^3 (5 μM) in the absence of Zn^{2+} at pH 7.4.

6c). Addition of Cu^{2+} caused the fluorescent quenching for all L^1−L^3.

Figure 7 compares the pH-dependent fluorescent changes of the free ligands L^3 and L^1 and the complexes ZnL^3 and ZnL^1 (5 μM, prepared in situ). The emission of ZnL^3 increased as pH was raised from pH 4 and reached a maximum plateau at pH 7−12, while L^3 emission reached a minimum plateau at pH 9−12. Accordingly, the maximum Zn^{2+} sensitivity (20 nM) with L^3 was obtained at pH > 9. By comparison, the maximum Zn^{2+} selectivity of L^1 was found at pH > 11.

Selective Metal Signaling of L^1−L^3. The fluorescence responses of L^1 and L^3 (5 μM) to Zn^{2+}, Cd^{2+}, Cu^{2+}, and other metal ions (5 μM) at pH 7.4 (1 mM HEPES with $I =$ 0.10 ($NaNO_3$)) and 25 °C are summarized in Figure 8. The Zn^{2+} selectivity is evident with L^3 at pH 7.4, while the Zn^{2+} selectivity with L^1 occurred only at higher pH. The dansyl-amide-pendant cyclen L^2 signaled Zn^{2+} and Cd^{2+} almost equally at pH 7.4.[3] The presence of an excess amount (up to 10−100 mM) of Mg^{2+}, Ca^{2+}, and K^+ had a negligible effect on the emission of 5 μM ZnL^3 or CdL^3. Counterions such as NO_3^-, ClO_4^-, SO_4^{2-}, F^-, Cl^-, Br^-, I^-, and HPO_4^{2-},[27] thymidyl anion,[21a,28] and barbiturate anion[27] (up to 5 mM) did not affect the emission intensity of ZnL^3, implying that these anionic ligands did not displace the Zn^{2+}−apical amine coordination. On the other hand, the 4-coordinate Zn^{2+}−cyclen complexes interact with these anions, especially barbiturate[27] and thymidyl anions[28] with a log K_{app} of 3−4.2 at pH 8 in aqueous solution.[29] L^3 is interesting in comparison with a recently reported structurally similar anion sensor, **10**, in which the pendant arylamine bound to Cd^{2+} ion was displaced by inorganic anions at pH 7.4.[30]

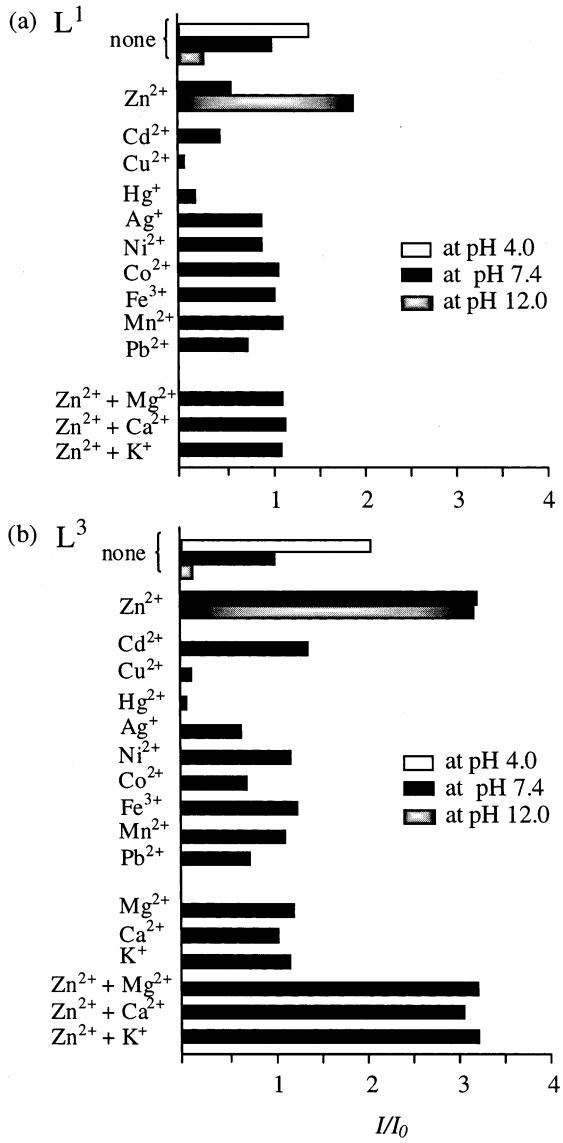
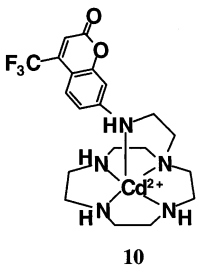


Figure 8. Relative fluorescence intensity at 416 nm of L^1 (a) and L^3 (b) responding to 1 equiv of metal ions at various pH values with $I = 0.1$ ($NaNO_3$) at 25 °C (excitation at 368 nm). I_0 is the emission intensity at 416 nm of L^1 and L^3 (5 μM) in the absence of metal ions at pH 7.4.



10

Kinetics of Zn^{2+} Complexation of L^2 and L^3. The kinetics of Zn^{2+} complexation of L^3 were compared those of L^2 in pH 7.4 aqueous solution with $I = 0.1$ ($NaNO_3$) at 25 °C. The initial reaction rates were followed by measuring

(27) (a) Koike, T.; Takamura, M.; Kimura, E. *J. Am. Chem. Soc.* **1994**, *116*, 8443−8449. (b) Fujioka, H.; Koike, T.; Yamada, N.; Kimura, E. *Heterocycles* **1996**, *42*, 775−787. (c) Kimura, E.; Koike, T. *J. Chem. Soc., Chem. Commun.* **1998**, 1495−1500. (d) Kimura, E. *Acc. Chem. Res.* **2001**, *34*, 171−179.

the increase in the emission of reacting solutions of L^2 and L^3 (2 or 5 μM) immediately after addition of Zn^{2+} (5, 20, and 100 μM). The second-order rate constant k_2 for the 1:1 $Zn^{2+}-L^3$ complexation was determined to be 4.6×10^2 M^{-1} s^{-1}, while k_2 values for ZnL^1 and ZnL^2 were 5.6×10 and 1.4×10^2 M^{-1} s^{-1}, respectively; i.e., the ZnL^3 formation was 8 and 3.3 times faster than ZnL^1 and ZnL^2 formation (see the Supporting Information). Thus, the insertion of an ethylamine pendant L^3 not only thermodynamically but also kinetically improved the Zn^{2+} complexation.

Conclusion. We have designed a new ligand, L^3, for an effective zinc(II) fluorophore and the equilibria and kinetics of Zn^{2+} complexation. The Zn^{2+}-responsive fluorescent emissions disturbed by protonation were studied in comparison with those of homologous L^1 and our previous zinc(II) fluorophore L^2. Several advantages of L^3 over L^1 or L^2 as a zinc(II) fluorophore were found: (1) The stable 1:1 complex of L^3 with Zn^{2+} was formed at lower pH (>6) than ZnL^1 and ZnL^2, allowing wider pH applications of L^3. (2) Upon complexation with Zn^{2+}, the fluorescent emission of L^3 linearly increased like that of L^2, while the emission of L^1 decreased at physiological pH. The detection limit of L^3 (5 μM) is $[Zn^{2+}] = 0.1$ μM at pH 7.4, which is almost equal to the sensitivity of L^2, and $[Zn^{2+}] = 20$ nM at pH 9.0. (3) Zn^{2+} ion was better sensed by L^3 than by L^1 at neutral pH. (4) L^2 responded to Zn^{2+} and Cd^{2+} undiscriminately at neutral pH, while L^3 was more selective to Zn^{2+} over Cd^{2+}. (5) The Zn^{2+} uptake emission response by L^3 was 8 and 3.3 times faster than those by by L^1 and L^2, respectively, in pH 7.4 aqueous solution. Thus, L^3 is thermodynamically, fluorometrically, and kinetically promising as a new zinc(II) fluorophore. Moreover, the present knowledge will be helpful in designing more useful macrocyclic zinc(II) fluorophores.

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Supporting Information Available: Figures S1 and S2 showing an FAB mass spectrum of ZnL^3, a theoretical distribution for $ZnL^3-H^+(C_{25}H_{34}N_5Zn)^+$, and the initial increase of fluorescence emission of L^2 and L^3 after addition of Zn^{2+} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) By addition of thymidine (dT) to 20 μM ZnL^1 at pH 7.4 (10 mM HEPES with $I = 0.1$ ($NaNO_3$)) and 25 °C, emission of ZnL^1 increased and its titration curve gave a 1:1 complexation constant log $K_{app}(ZnL^1{-}dT^-)$ ($K_{app}(ZnL^1{-}dT^-) = [ZnL^1{-}dT^-]/[ZnL^1]_{free}[dT]_{free}$ (M^{-1})) of 4.4 ± 0.2. Addition of HPO_4^{3-} to 20 μM L^1 caused colorless precipitations.

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