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A Synthetically Simple, Click-Generated Cyclam-Based Zinc(II) Sensor

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A cyclam-based macrocyclic sensor has been prepared using synthetically simple "click" chemistry to link a fluorophore to the macrocyclic receptor. This sensor shows high selectivity for Zn(II) over a range of other metals, providing a significant enhancement of fluorescence intensity over a wide pH range. As such, this is the first cyclam-based sensor demonstrated to be selective for Zn(II) and is the first example of a triazole being used as a coordinating ligand on an azamacrocycle. The sensor can access biologically available zinc in mammalian cells, sensing the Zn(II) flux that exists during apoptotic cell death.

Introduction

Zinc is the second most abundant d-block metal in the human body, playing a critical role in enzyme regulation, structure and function, neural signal transmission, and gene expression.[1] While most zinc is tightly bound in proteins, "mobile" pools of zinc exist in certain mammalian organs such as the brain and pancreas, which are carefully regulated,[2] the disruption of which is associated with a number of disease states including types I and II diabetes, Parkinson's disease, epilepsy, and certain cancers.[2a,3] Moreover zinc is now recognized as an important factor in the regulation of apoptosis.[4] Given that the d^{10} electronic configuration of Zn(II) renders it spectroscopically silent, the majority of recent efforts to develop effective small molecule sensors for Zn(II) have focused on the retardation of photoinduced electron transfer (PET) developed by de Silva, in which metal binding occurs at a site in close proximity to an appropriate fluorophore and switches on fluorescence.[5] To date, a great many sensors have been reported,[6] perhaps most notably by Lippard et al.[7] There continues to be considerable interest

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in the development of more effective analogues to further our understanding of the plethora of in vivo settings in which zinc is known to play a crucial role.[8]

Despite the extensive literature of cyclen-based sensor agents,[4,6c,9] rather surprisingly, there are very few examples of analogous small molecule cyclam-based fluorescent sensors, and these have not been shown to display selectivity for Zn(II).[5e,10] We have recently become interested in the incorporation of biological binding motifs into azamacrocyclic scaffolds,[11] in particular, the use of "click" chemistry[12] to construct sensors based on this design. We have demonstrated that the triazole resulting from the click reaction is a competent coordinating motif for "scorpion-like" complexes,[10a,13] and that perturbation of the interaction between the triazole donor and the metal can act as a novel means of detecting analyte binding spectroscopically.[11b] This approach seemed promising for the development of a PET Zn(II) sensor. The triazole here would resemble histidine ligands that strongly bind zinc ions in a range of enzymes such as carbonic anhydrase.[14]

Experimental Section

General Information. All reagents were purchased from Sigma-Aldrich and used without further purification. All reactions were carried out using commercial-grade solvents. 1,4,8-Triboc-cyclam and compound **2** were prepared as described in the literature.[15−17] Silica gel chromatography was carried out using BDH silica gel for flash chromatography as the stationary phase and EtOAc/Pet. Spirit 30−40 °C as eluents.

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, CD_3CN, or DMSO-*d*6 with a Jeol 270 MHz spectrometer. ^{1}H chemical shifts are reported in parts per million relative to the residual proton signal of the deuterated solvents. Coupling constants (*J*) are reported in Hertz. ^{13}C chemical shifts are reported in parts per million relative to the carbon signal of the solvents. IR spectra were recorded with a Shimadzu FTIR-8300. Fluorescence emission spectra were recorded with a Jobin Yvon Horiba FluoroMax-3 in a 1-cm-path-length cell.

Electrospray ionization mass spectra were obtained from the EPSRC National Mass Spectrometry Service, University of Wales, Swansea, using either a Waters ZQ400 or a Micromass Quattro II. Accurate masses were recorded with either a Finnigan MAT900 or MAT 95 using polyethylenimine as the reference.

11-Prop-2-ynyl-1,4,8,11-tetraaza-cyclotetradecane-1,4,8-tricarboxylic Acid Tri-*tert*-butyl Ester (1). To a solution of 1,4,8-triboc-cyclam[15] (250 mg, 0.5 mmol) in CH_3CN (15 mL) were added Na_2CO_3 (1 mmol, 212 mg) and propargyl bromide (0.6 mmol, 67 μL), and the mixture was stirred at reflux (85 °C) overnight. The insoluble salts were filtered and the solvent removed in vacuo. The crude material was purified by flash chromatography on silica gel (EtOAc/Pet. Spirit 7:3) to give **1** as a white solid (211 mg, 78% yield): mp 47−49 °C. ν_{max} (CH_2Cl_2)/cm^{-1}: 3301, 2135, 1685. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 3.48−3.15 (m, 14H), 2.71−2.58 (m, 2H), 2.49 (t, J = 5.4 Hz, 2H), 2.14 (s, 1H), 1.98−1.77 (m, 2H), 1.76−1.60 (m, 2H), 1.44 (s, 27H). ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 155.8, 155.5, 79.6, 79.5, 77.9, 73.2, 53.0, 51.9, 50.7, 48.0, 47.5, 46.9, 46.7, 44.8, 41.9, 28.5, 25.5. MS (ESI): *m*/*z* 539 [(M + H)$^+$]. HRMS (ES) calcd. for $C_{28}H_{51}N_4O_6$ (M + H)$^+$: 539.3803. Found: 539.3800.

6-Azido-2-ethyl-benzo[de]isoquinoline-1,3-dione (2).[16,17] 4-Bromo-1,8-naphthalic anhydride (500 mg, 1.8 mmol) and ethyl amine (70% solution in H_2O, 172 μL, 2.2 mmol) were refluxed in dioxane (30 mL) for 7 h. The solution was cooled to room temperature and poured into water to precipitate out a solid, which was collected by filtration, washed with water, and dried in vacuo to give a cream-colored solid (450 mg, 82%). A mixture of the solid so obtained (420 mg, 1.4 mmol) and sodium azide (449 mg, 6.9 mmol) in *N*-methylpyrrolidinone (6 mL) was heated at 110 °C for 1.5 h. The reaction mixture was diluted with water and extracted with EtOAc (3 × 20 mL). The organic phase was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (EtOAc/Pet. Spirit 4:6) to give the desired product (**2**) as a dark brown solid (182 mg, 50%). Spectroscopic data were identical to those reported in the literature.[16,17]

11-[1-(2-Ethyl-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)-1H-[1,2,3]triazol-4-ylmethyl]-1,4,8,11tetraaza-cyclotetradecane-1,4,8-tricarboxylic Acid Tri-*tert*-butyl Ester (3). To a solution of **1** (269 mg, 0.5 mmol) in THF/H_2O (7/3, 10 mL) were added **2** (133 mg, 0.5 mmol), $CuSO_4 \cdot 5H_2O$ (5 mol%, 6.2 mg, 0.025 mmol), and sodium ascorbate (10 mol %, 9.9 mg, 0.05 mmol) under N_2. The solution was stirred at room temperature overnight. Saturated NH_4Cl was added (20 mL), and THF was evaporated in vacuo. The aqueous phase was extracted with DCM (3 × 20 mL); the organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude material was filtered through a short plug of silica (EtOAc/Pet. Spirit 1:1) to give **3** as a yellow solid (195 mg, 82%): mp = 91−93 °C. ν_{max} (CH_2Cl_2)/cm^{-1}: 3055, 1662, 1647. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.76−8.66 (m, 2H), 8.30−8.22 (m, 1H), 8.02−7.80 (m, 3H), 4.27 (q, J = 7.0 Hz, 2H), 3.94 (s, 2H), 3.48−3.24 (m, 12H), 2.78−2.68 (m, 2H), 2.62−2.52 (m, 2H), 1.96−1.84 (m, 2H), 1.84−1.72 (m, 2H), 1.48−1.30 (m, 30H). ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 163.3, 162.8, 155.8, 155.5, 144.5, 138.1, 131.9, 130.6, 129.5, 128.9, 128.5, 126.3, 125.1, 123.7, 123.5,

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122.8, 79.6, 77.5, 51.4, 50.0−46.0 (m), 45.6, 35.7, 28.4, 26.7, 13.4. MS (ESI): *m/z* 805 [(M + H)$^+$]. HRMS (ESI) calcd for $C_{42}H_{61}N_8O_8$, (M + H)$^+$: 805.4607. Found: 805.4618.

2-Ethyl-6-[4-(1,4,8,11-tetraaza-cyclotetradec-1-ylmethyl)-[1,2,3]triazol-1-yl]-benzo[de]isoquinoline-1,3-dione (4). Compound **3** (190 mg, 0.2 mmol) was dissolved in a solution of TFA (20%) in DCM (5 mL), and the yellow solution was stirred overnight at room temperature. Saturated $NaHCO_3$ was added to neutralize the excess of TFA, and the mixture was extracted with DCM (3 × 20 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo to give the desired product (**4**) as a yellow solid (105 mg, 88%): mp = 65−67 °C. ν_{max} (CH_2Cl_2)/cm^{-1}: 3055, 1662, 1593. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.65 (d, J = 7.9 Hz, 2H), 8.15 (d, J = 8.6 Hz, 1H), 7.98 (s, 1H), 7.91 (d, J = 7.9 Hz, 1H), 7.79 (dd, J = 8.6, 8.4 Hz, 1H), 4.22 (q, J = 7.0 Hz, 2H), 3.50−2.70 (m, 19H), 2.20−1.80 (m, 4H), 1.31 (t, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 163.4, 162.8, 144.6, 137.8, 132.1, 130.6, 129.2, 128.9, 128.7, 126.5, 125.1, 124.2, 124.1, 122.9, 77.3, 56.3, 54.5, 49.4, 48.8, 48.5, 47.8, 47.1, 45.8, 45.6, 35.8, 25.7, 23.4, 13.3. MS (ESI): *m/z* 505 [(M + H)$^+$], 282, 241, 226. HRMS (ESI) calcd for $C_{27}H_{37}N_8O_2$, (M + H)$^+$: 505.3034. Found: 505.3033.

[Zn(4)](ClO$_4$)$_2$. To a solution of **4** (150 mg, 0.29 mmol) in MeOH (6 mL) was added $Zn(ClO_4)_2 \cdot 6H_2O$ (111 mg, 0.29 mmol). A pale yellow precipitate started to form almost immediately. The mixture was stirred overnight at room temperature, and the solid formed was then filtered, washed with cold MeOH (ca. 3 mL), and dried in vacuo to give the desired product [Zn(**4**)]$(ClO_4)_2$ as a pale yellow solid (129 mg, 65%). ν_{max} (CH_2Cl_2)/cm^{-1}: 1710, 1666, 1099, 1041, 750. ^{1}H NMR (CD_3CN, 270 MHz): δ 8.74−8.62 (m, 2H), 8.42−8.38 (m, 1H), 8.34−8.18 (m, 1H), 8.08−7.88 (m, 2H), 4.48 (d, J = 18.4 Hz, 1H), 4.24−4.12 (m, 2H), 4.01 (d, J = 18.4 Hz, 1H), 3.40−2.70 (m, 14H), 2.64−2.48 (m, 1H), 2.44−2.24 (m, 1H), 2.14 (s, 3H), 1.96−1.70 (m, 4H), 1.36−1.24 (m, 3H). ^{13}C NMR (DMSO-*d*6, 67.5 MHz): δ 163.5, 162.9, 142.9, 138.1, 132.1, 131.0, 129.8, 129.4, 128.9, 127.6, 126.2, 124.8, 124.0, 123.2, 55.4, 51.8, 51.3, 49.1, 48.7, 46.4, 45.7, 45.1, 44.8, 44.6, 35.6, 25.4, 22.6, 13.6. MS (ESI): *m/z* 667 [M$^+$], 605, 567. HRMS (ESI) calcd for $C_{27}H_{36}N_8O_6ClZn$, (M)$^+$: 667.1732. Found: 667.1732. The fluorescence quantum yield of the zinc complex ([Zn(**4**)]$(ClO_4)_2$) in 50 mM HEPES buffer solution (I = 0.1 $NaNO_3$) was found to be 0.04. Fluorescence quantum yield was determined by comparison with standard anthracene (Φ = 0.27 in ethanol).[18]

Flow Cytometeric Analysis of 4 in Primary Murine Cells. Single-cell suspensions of freshly isolated murine thymocytes from C57BL/6 mice were prepared by pressing mouse thymi between the frosted ends of two sterile glass microscope slides. Tissue clumps were removed by passage over a nylon filter. Single-cell thymocytes were cultured in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (JRH) in the presence or absence of 1 μM staurosporine (Sigma) for 24 h to induce cell death. Thymocytes were aliquoted into 96-well U-bottom plates in triplicate, and incubated with 50, 100, 250, or 500 μM **4** for 1 h at 37 °C. Cells were washed in 100 μL of PBS by centrifugation at 1200 rpm for 5 min, incubated in AnnexinV-biotin (BD Biosciences) for 45 min at 4 °C and then streptavidin-APC-Cy7 for 45 min at 4 °C, washed again, and resuspended in saline containing 7.5 nM propidium iodide (Invitrogen). The fluorescence of **4** in primary thymocytes was detected using flow cytometry using a Becton Dickenson LSRII multicolor flow cytometer, where the fluorescence was excited with a 350-nm-wavelength UV laser and the emission detected at 450 nm (FL8) with a 50-nm-band-path-width filter. At this wavelength, the natural autofluorescence of murine thymocytes is simultaneously detected. Data for 30 000 events (cells) were collected and analyzed using the CellQuestPro software (Becton Dickenson), version D.9.9f4b. Data (two-color dotplots and histograms of mean fluorescence intensity) were imported into the Canvas9 drawing software, version 9.0.2 (ACD Systems Ltd.), to generate composite figures and were saved as jpg files.

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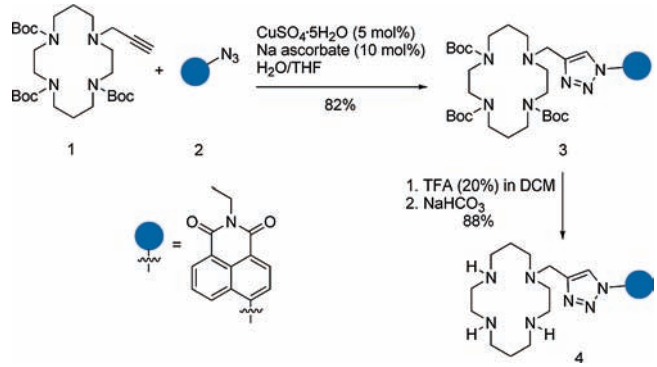
Scheme 1. Preparation of the "Click"-Generated Cyclam Fluorophore



Results and Discussion

We chose to develop a modular ligand synthesis based on established chemistry utilizing a "click" cycloaddition to incorporate the fluorophore. In this way, we anticipated that a range of fluorophores could ultimately be incorporated into the sensor without detriment to the efficacy of the system, permitting the generation of a library of Zn(II) sensors with different excitation and emission wavelengths. Fluorophore **2** (Scheme 1) was adopted as our model system since it is available in two steps from commercially available 4-bromo-1,8-naphthalic anhydride, a fluorophore used in the synthesis of other sensors.[6a,g,17] Similarly, Boc-protected cyclam **1** may be conveniently accessed in two steps from triBoc-protected cyclam[15] and propargyl bromide and undergoes a smooth and high-yielding [3 + 2] "click" cycloaddition with fluorophore **2**. Deprotection of the Boc-carbamate **3** with TFA followed by neutralization of the resultant salt afforded the novel water-soluble cyclam derivative **4** in excellent overall yield.

The mode of coordination of **4** with Zn(II) was investigated by monitoring the fluorescent emission at 407 nm (350 nm excitation) upon the titration of a 300 μM solution of Zn(II) into a 10 μM solution of **4** in a HEPES buffer at pH 7.0. The significant (6-fold) enhancement of the fluorescence emission up to the addition of 1 equiv of Zn(II) is entirely consistent with the formation of the 1:1 [ML] complex anticipated (Figure 1), and the stability constant was estimated to be 2.30 × 10^7 M^{-1} (R = 0.981) by using nonlinear least-squares analysis.[19] Single-crystal X-ray crystallography confirmed the connectivity of the structure, but the quality of the data was not sufficient for publication purposes (see the Supporting Information). The detection limit of **4** is ca.

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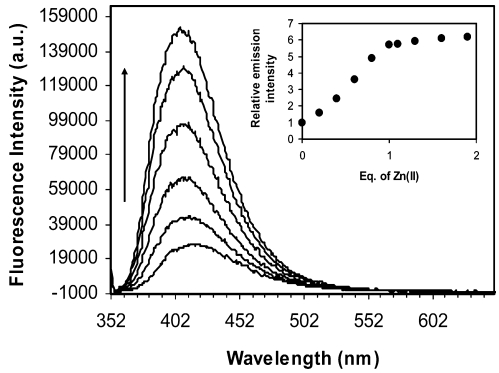

Figure 1. Fluorescence emission response upon titration of 300 μM zinc(II) into a 10 μM solution of **4** at 25 °C and pH 7 (1.0 mM HEPES buffer). Inset: the increase in fluorescence emission intensity relative to the number of equivalents of Zn(II) added.

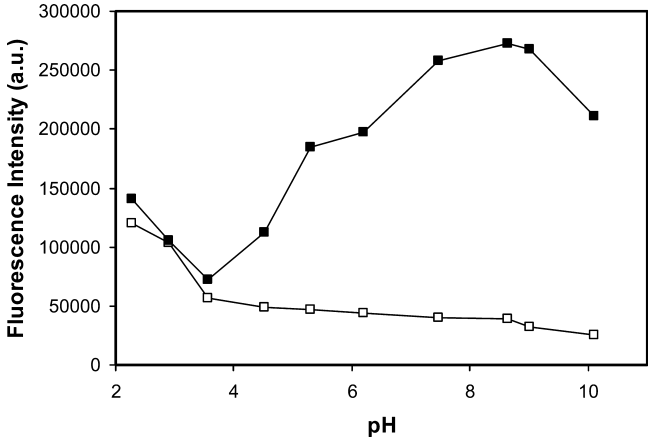

Figure 2. Fluorescence emission of ligand **4** in the absence (□) and presence (■; ([Zn(**4**)](ClO$_4$)$_2$)) of Zn(II) over a range of pH's.

2 μM Zn(II), which is significantly lower than the physiological concentration of 10−300 mM estimated in zinc pools.[20]

One attraction of the click-generated scorpion ligand is the lack of an acidic proton requiring deprotonation to coordinate to Zn(II) and thus switch on fluorescence (as required for other cyclen-based zinc sensors[9b−d] employing a scorpionate pendant arm). As a result, **4** showed an excellent fluorescence response over a wide range of pH's with a significant enhancement being observed compared to free ligand **4**, Figure 2. At low pH, the responses of **4** and its Zn(II) complex were comparable, presumably as a result of protic quenching of PET. However, at physiologically relevant pH, the complex displayed a significant enhancement over the free ligand.

An important feature of any prospective Zn(II) sensor is its ability to detect Zn(II) selectively over other physiologically relevant divalent cations. In this regard, **4** upholds exceptional selectivity for Zn(II), Figure 3, and is comparable to the most effective sensors reported. In contrast to many other Zn(II) sensors,[4,9b−c] excellent selectivity is also seen over Cd(II). The response of **4** to Zn(II) is extremely rapid, and a maximal fluorescence response is almost immediately observed upon addition of the metal. In the case of Cd(II),

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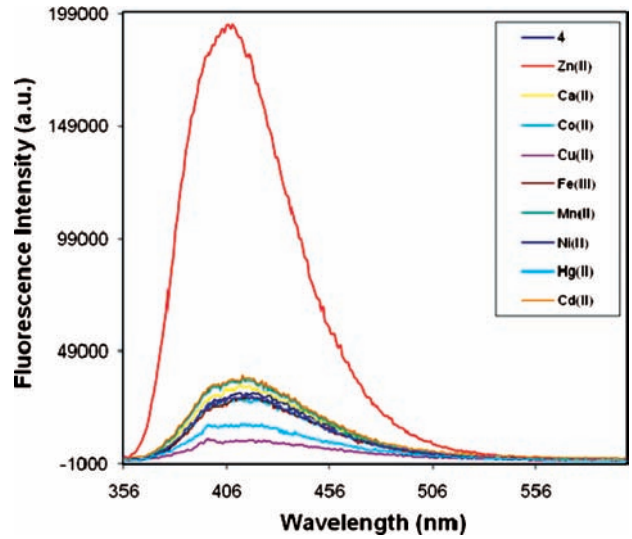

Figure 3. Fluorescence emission of **4** (10 μM) in the presence of a number of other metals (10 μM) at pH 7.0 in a HEPES buffer ca. 5 min after the addition of the metal to the ligand.

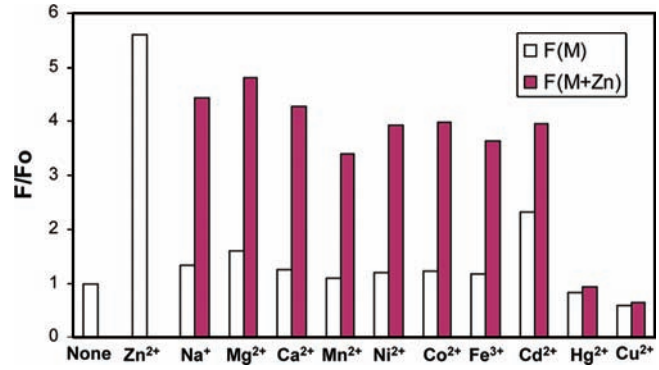

Figure 4. Competitive binding experiments of **4** in which the competing metals (50 μM) were added to **4** (10 μM) followed by Zn(II) (10 μM) in a HEPES buffer at pH 7.0.

the response over the same time scale is negligible, and after 5 min, there is very little difference between the free ligand and the metal complex. It is only after prolonged mixing with Cd(II) over a 24 h period that the maximal response is achieved, which is still more than 3 times less than that immediately observed for Zn(II). Interestingly, it was recently proposed that the selectivity of a triazole-based sensor for Zn(II) over Cd(II) was due to the size of the chelate ring formed upon coordination of the metal (and the resultant strain): a six-membered chelate ring showed excellent Cd(II) selectivity, while a smaller five-membered chelate ring system did not.[6h] Our observations suggest that factors beyond the size of the chelate are important in determining metal ion selectivity.

Another important feature in a sensor is its ability to function in competition with other relevant metals. Thus, 5-fold molar excesses of a number of metal ions were added to a solution of **4** prior to the addition of 1 equiv. of Zn(II) approximately 20 min later, and their emission spectra were recorded (Figure 4). In all cases, the addition of Zn resulted in an increase of the fluorescence response of the solution, although the maximum fluorescence observed was lower than that for the solution containing only **4** and Zn(II). In the case

of the physiologically relevant Mg(II), Ca(II), and Na(I), this was relatively minor. Furthermore, despite the high stability constants that are observed for cyclam with d-block metals, this reduction in intensity was also relatively minor for Mn(II), Fe(III), Co(II), Ni(II), and Cd(II). In the case of Cu(II) and Hg(II), however, it is apparent that the initially formed complex is too stable for it to be displaced by Zn(II), as both samples displayed quenching of the emission; although in both cases, a slight increase in the emission in the presence of Zn(II) was observed.

We next examined the sensor in a cellular system, using freshly isolated murine thymocytes. Because Zn(II) is a factor in the regulation of apoptosis,[4] we examined the fluorescence emission in three sample preparations: thymocytes incubated in vitro for 24 h in the presence or absence of the apoptosis inducer staurosporine and a 1:1 mixture of cells cultured with and without staurosporine. To determine whether **4** was able to fluoresce as a result of the Zn(II) flux that occurs in apoptotic cells, thymocytes were incubated in various concentrations of **4** and then stained with annexinV-biotin and streptavadin-APC-Cy7, together with propidium iodide (PI). AnnexinV is a marker of apoptotic cells, and it is detected by staining the cells with AnnexinV-biotin then streptavidin-APC-Cy7 and detecting APC-Cy7 fluorescence at 780 ± 60 nm (excited by a red 633 nm laser), whereas PI is a fluorescent dye that permeates only dead cells and is detectable at 695 ± 40 nm (after excitation with the blue 488 nm laser). Therefore, this staining regime enables the discrimination of live cells (annexinV-negative, PI-negative) from dead cells (annexinV-positive, PI-positive),[21] and simultaneous detection of fluorescence from **4** detected at 450 ± 50 nm (excited by a UV 350 nm laser). The flow cytometry data indicate that live thymocytes have a background level of fluorescence when detected at 450 nm (autofluorescence, R2, Figure 5A), whereas dead cells have reduced autofluorescence (R1, Figure 5A). Nevertheless, there was a concentration-dependent increase in fluorescence from **4** (Figure 5B), with the greatest increase in fluorescence being in the dead cells (Figure 5C). Since we have established that Zn(II) selectively induces fluorescence in the presence of **4**, these data strongly suggest that **4** fluoresces as a result of exposure to increased intracellular levels of Zn(II). In fact, **4** is exquisitely sensitive to endogenous Zn(II) because the addition of exogenous 25 mM zinc sulfate and 2 mM sodium pyrithione was not required for detection of the fluorescence (data not shown), unlike some other fluorescent zinc sensors reported to date.[4]

Conclusions

The zinc sensor described has a number of important features. First, while cyclen-based systems have been used extensively in sensing, there are essentially only two applications to date of cyclam-based zinc sensors,[5e,10c] neither of which are structurally similar to ours (one being a dendrimer). In neither case was selectivity for zinc over other ions reported. The sensor reported here is hence the first zinc-selective sensor based on cyclam, and from this point of view, the level of selectivity we observe for zinc is surprising. Our results also reveal something of the mechanism of this selectivity. A previously reported zinc sensor displayed selectivity over cadmium, and it was suggested that this was due to a six-membered chelate ring, since an analogous ligand with a smaller five-membered ring did not provide selectivity.[6h] In contrast, our sensor, containing a five-membered chelate ring, displays exceptional zinc selectivity over cadmium, and this suggests that other effects (e.g., conformational effects of the azamacrocyclic ring) will be important to consider in the design of future sensors. Our sensor also displays zinc selectivity over a broad pH range, presumably because the coordinating ligand does not need

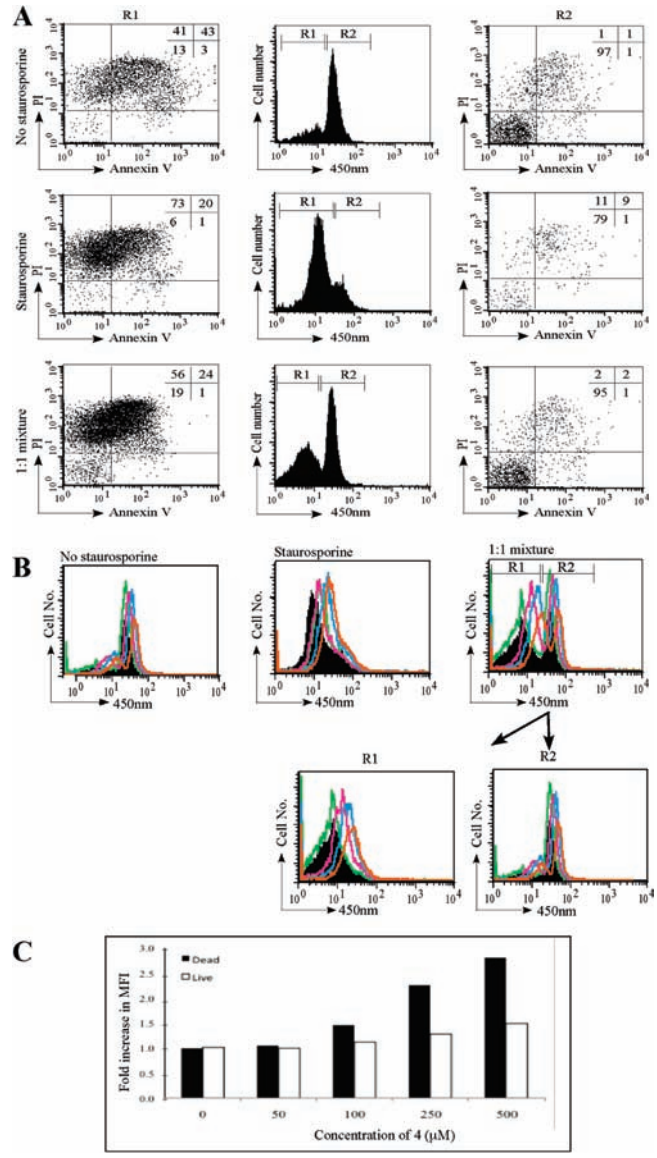

Figure 5. (A) Priopidium iodide and annexin-V dot plots of live (R2) and dead (R1) populations of murine thymocytes cultured in the presence or absence of staurosporine and gated first on autofluorescence at 450 nm. (B) Fluorescence of **4** at various concentrations (green 50 μM, pink 100 μM, blue 250 μM, orange 500 μM), overlaid against the absence of **4** (black-filled histogram), detected at 450 nm. (C) Fold change of mean fluorescence intensity (MFI) of **4** at various concentrations (as above) in live (unfilled) and dead (black) gated thymocytes detected at 450 nm.

(21) Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutellingsperger, C. *J. Immunol. Methods* **1995**, *184*, 39–51.

to be deprotonated for the sensor to be active. Previously, zinc sensors have been found to be highly pH-sensitive,[9b−d] or this has not been addressed.[6h]

The synthetic design of this sensor is modular, in that it is simple to attach a number of fluorophores to the various azamacrocycles using the click approach. To the best of our knowledge (SciFinder searches on this and related structures), this is the first example of a triazole being used as a scorpionate ligand on an azamacrocycle, and one can envisage other uses for such a generic system more generally in coordination chemistry and chemical sensing. Lastly, we have shown that the sensor is competent in the detection of cellular levels of zinc during apoptosis without the need for added extracellular zinc, unlike many previously reported sensors (e.g., ref 4). This may make this sensor design attractive for in vivo applications.

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Supporting Information Available: Nuclear magnetic resonance spectra for compounds synthesized, a nonlinear curve fitting for [**4**·Zn], and the single-crystal X-ray structure for $[Zn(\mathbf{4})](ClO_4)_2$ together with its mass spectrometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

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