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The Rhodafluor Family. An Initial Study of Potential Ratiometric Fluorescent Sensors for Zn^{2+}

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A new class of ratiometric Zn^{2+} sensors that employ a hybrid fluorescein and rhodamine fluorophore has been designed, and two members of the rhodafluor family of sensors, RF1 and RF2, have been synthesized. The preparation of RF1 (9-(*o*-carboxyphenyl)-2-chloro-6-[bis(2-pyridylmethyl)amino]-3-xanthanone, Rhodafluor-1), uses conventional synthetic methods. Elaboration of the RF1 synthesis in an effort to enhance the Zn^{2+} affinity was unsuccessful, so palladium-catalyzed aryl amination was applied to prepare RF2 (1-[9′-(*o*-carboxyphenyl)-6′-amino-2′-chloro-3′-xanthanone]-4,10-(diethyl)-7-(2-pyridylmethyl)-1,4,7,10-tetraazacyclododecane, Rhodafluor-2). The key step in the synthesis of RF2 is coupling of a triprotected tetraazamacrocycle (cyclen) to 3-bromoanisidine. RF2 binds Zn^{2+} with a dissociation constant of 13.5 μM accompanied by an ~50% increase in quantum yield. Although only small shifts in absorption wavelength were observed, because protonation of the amino nitrogen atoms of the macrocycle prevents the uncomplexed sensor from adopting the desired mesomer, the intensity doubling makes the probe of value for immediate application in situations where our previous tight binding (<1 nM) sensors are inadequate.

Introduction

Studying the function of free Na^+, K^+, Ca^{2+}, Mg^{2+}, and Zn^{2+} in biological systems can be challenging because these metal ions lack spectroscopic properties. Their investigation can be facilitated by the use of fluorescent probes,[1−3] however. Fluorescence-based sensors consist of a receptor capable of selectively binding the species of interest, linked to a fluorophore that undergoes a change in optical emission upon analyte binding.[4] Since the development of efficient synthetic techniques is required to access desired sensor molecules, organic chemistry plays a crucial role in the service of biological application.

The most common class of fluorescent sensors for metal ions is based on photoinduced electron transfer (PET) quenching mechanisms. PET sensors consist of a receptor covalently linked to a fluorophore. In the absence of analyte, electrons localized on a donor atom of the receptor participate in back ET with the excited state of the fluorophore, quenching emission. Upon complexation of a metal ion, the electronic structure of the receptor changes, interrupting PET and restoring fluorescence.[4] Such "off−on" fluorescent probes are often employed as biosensors because typically they are easy to prepare, emit an intense fluorescent signal, and have well-understood photophysics.

In an effort to study the function of Zn^{2+} in neurobiology, we have designed and reported on a series of Zn^{2+}-specific PET sensors.[5−8] ZP1, ZP2, and ZP4 are members in a series of fluorescein-based sensors designed to induce a positive fluorescence response upon Zn^{2+} complexation. Although these dyes offer several advantages over traditional Zn^{2+} sensors, they also illustrate the challenges of using PET-based probes for biological imaging. Metal-binding atoms in the receptor that are responsible for PET quenching can be susceptible to proton-induced fluorescence under physi-

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ological conditions. The magnitude of the ZP sensor fluorescence response to Zn^{2+} is greater than that to protons, but background fluorescence from the protonated probe interferes with the quantitation of zinc concentration. Ideally, the fluorescence of PET sensors is independent of pH, the probes being nonemissive in the absence of analyte. Without a fluorescence signal from free probe, however, monitoring of the distribution of sensors in cells is difficult. As with ZP1 and ZP2, which tend to localize in vesicles,[5] sensors may disperse unevenly in a dynamic environment like biological tissue because of their chemical properties.[9] Imaging and measurements may therefore reflect the distribution of the free probes rather than analyte.

One possibility to circumvent the obstacles associated with intensity probes is to alter the sensing strategy. Integration of a donor group of the receptor into the π-system of a fluorophore produces sensors that undergo a spectral shift in the excitation and/or emission wavelength upon metal ion binding. The key mechanistic feature of such ratiometric probes is the decoupling of donor electrons from resonance with the fluorophore, an event that changes the electronic structure. Several ratiometric sensors for Ca^{2+} exist,[1,9] yet only a handful of Zn^{2+} probes have been reported,[10,11] none of which is based on a fluorescein scaffold. A ratiometric Ca^{2+} sensor utilizing a hybrid fluorescein and rhodamine fluorophore was proposed originally[12] and reported previously.[13] Although this sensor was successfully obtained, the laborious synthesis is unattractive and lacks versatility. In the present work, we describe methodology for preparing fluorescein/rhodamine hybrid sensors by applying aryl−azamacrocycle coupling[14] to make a first generation ratiometric sensor for Zn^{2+}.

Experimental Section

Materials and Methods. Acetonitrile, dichloromethane, dichloroethane (DCE), and triethylamine were distilled from CaH_2 under nitrogen. Chloroform was passed through a column of basic aluminum oxide and dried over 3 Å molecular sieves. Toluene was distilled from Na/benzophenone ketyl. Methanol was distilled from Mg/I_2. $CDCl_3$ was dried over 3 Å molecular sieves. 2′-Carboxy-5-chloro-2,4-dihydroxybenzophenone (**4**) was prepared as previously described.[6] All other reagents were purchased and used as received. Flash column chromatography was performed with silica gel-60 (230−400 mesh) or Brockman I activated basic aluminum oxide (150 mesh). Thin layer chromatographic (TLC) analysis was performed with Merck F254 silica gel-60 or Merck F254 aluminum oxide-60 plates and viewed by UV light or developed with ceric ammonium molybdate, ninhydrin, or iodine stain. NMR spectra were recorded on a Varian 500 MHz or Mercury 300 MHz spectrometer at ambient probe temperature, 283 K, and referenced to the internal ^1H and ^{13}C solvent peaks. Infrared spectra were recorded on a BTS 135 or an Avatar 360 FTIR instrument as KBr pellets or thin films on NaCl plates. Electrospray ionization (ESI) mass spectrometry was performed in the MIT Department of Chemistry Instrumentation Facility (DCIF) with the use of *m*-nitrobenzyl alcohol as the matrix.

***N,N*-Bis(2-pyridylmethyl)-*m*-anisidine (2).** Picolyl chloride hydrochloride (12.0 g, 73.4 mmol) was dissolved in 3 mL of water, and 18 mL of 5 N NaOH was added to give a pink solution. An additional 18 mL of 5 N NaOH was added to the vigorously stirred solution, after *m*-anisidine (**1**, 1.8 mL, 16.0 mmol) was combined with the solution of picolyl chloride. An aliquot of cetyltrimethylammonium chloride (250 μL, 25 wt % in water) was added as a phase transfer catalyst (PTC), and the reaction mixture was stirred vigorously under Ar. Additional picolyl chloride hydrochloride (5.6 g, 34.3 mmol) was added to the solution after 48 h, and after 144 h, an additional portion of picolyl chloride hydrochloride (10.0 g, 61.2 mmol) and 15 mL of 5 N NaOH. After a total reaction time of 11 days, the product was extracted into CH_2Cl_2 and dried over $MgSO_4$, to give a brown solid after solvent removal. Flash chromotography on basic alumina with a solvent gradient (24:1 to 4:1 CH_2Cl_2/EtOAc) yielded the product as a yellow solid (1.72 g, 35.2%). TLC: $R_f = 0.33$ (4:1 CH_2Cl_2/EtOAc). ^1H NMR ($CDCl_3$, 500 MHz): δ 3.69 (3 H, s), 4.85 (4 H, s), 6.27 (1 H, t, $J = 2.5$ Hz), 6.30 (2 H, tt, $J = 2.0, 7.5$ Hz), 7.08 (1 H, t, $J = 8.0$ Hz), 7.17 (2 H, t, $J = 5.0$ Hz), 7.31 (2 H, d, $J = 7.5$ Hz), 7.67 (2 H, t, $J = 7.5$ Hz), 8.61 (2 H, dd, $J = 1.0, 5.0$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 55.23, 57.5, 99.26, 102.29, 105.75, 120.94, 122.20, 130.19, 137.01, 148.88, 158.93, 160.94. FTIR (thin film, cm^{-1}): 3418, 1612, 1591, 1500, 1471, 1434, 1346, 1264, 1201, 1168, 755. HRMS (ESI): calcd for MH^+, 306.1606; found, 306.1606.

***N,N*-Bis(2-pyridylmethyl)-3-aminophenol (3).** A solution of *N,N*-bis(2-pyridylmethyl)-*m*-anisidine (**2**, 500 mg, 1.64 mmol) in 20 mL of CH_2Cl_2 was frozen with liquid N_2, and 50 mL of 1.0 M BBr_3 (50 mmol) in CH_2Cl_2 was added via a cannula. The solution was allowed to warm slowly to room temperature and stirred under Ar for 40 h. The reaction mixture was chilled to −40 °C by a 2-propanol/dry ice bath, and MeOH was added slowly to quench the excess BBr_3. The quenched reaction mixture was diluted with ∼300 mL of water and boiled for 45 min. After the aqueous solution was cooled, neutralized to pH ∼6.5 with saturated $NaHCO_3$, and saturated with KCl, the product was extracted into CH_2Cl_2 to give a red solid after solvent removal. Flash chromotography on basic alumina (4:1 CH_2Cl_2/EtOAc) yielded the product as a yellow solid (190 mg, 39.8%). TLC: $R_f = 0.27$ (17:3 CH_2Cl_2/EtOAc). ^1H NMR ($CDCl_3$, 500 MHz): δ 4.81 (4 H, s), 6.12 (1 H, t, $J = 2.0$ Hz), 6.23 (1 H, dd, $J = 2.5, 8.0$ Hz), 6.30 (1H, dd, $J = 2.0, 8.0$ Hz), 7.04 (1 H, t, $J = 8.0$ Hz), 7.11 (2 H, t, $J = 5.0$ Hz), 7.29 (2 H, d, $J = 8.0$ Hz), 7.64 (2 H, td, $J = 1.5, 7.5$ Hz), 8.34 (2 H, d, $J = 4.5$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 57.28, 99.74, 104.26, 105.56, 121.19, 122.45, 130.69, 137.61, 149.06, 149.36, 158.56, 158.88. FTIR (thin film, cm^{-1}): 3413, 1613, 1595, 1502, 1438, 1355, 1191, 756. HRMS (ESI): calcd for MH^+, 292.1450; found, 292.1444.

9-(*o*-Carboxyphenyl)-2-chloro-6-[bis(2-pyridylmethyl)amino]-3-xanthanone (5, Rhodafluor-1, RF1). *N,N*-Bis(2-pyridylmethyl)-3-aminophenol (**3**, 300 mg, 1.03 mmol) and 2′-carboxy-5-chloro-2,4-dihydroxybenzophenone (**4**, 295 mg, 1.01 mmol) were combined in 5 mL of methanesulfonic acid (CH_3SO_3H). The resulting dark red solution was stirred for 48 h at 70 °C. The reaction mixture was diluted with 250 mL of water, chilled to 0 °C, and slowly neutralized with saturated $NaHCO_3$. The aqueous mixture was extracted thoroughly with CH_2Cl_2, and the combined organic

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extracts were dried over $MgSO_4$ to give a red solid after filtration and solvent removal. Flash chromatography on silica (93:7 $CHCl_3$/MeOH) yielded the product as a red solid (322 mg, 57.1%). TLC: $R_f = 0.47$ (9:1 $CHCl_3$/MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.84 (4 H, s), 6.43 (2 H, d, $J = 10.5$ Hz), 6.56 (1 H, d, $J = 8.5$ Hz), 6.73 (2 H, s), 7.14−7.24 (5 H, m), 7.56−7.69 (4 H, m), 8.02 (1 H, d, $J = 7.5$ Hz), 8.54 (2 H, d, $J = 5.0$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 56.57, 98.94, 103.81, 108.25, 109.70, 111.85, 121.25, 122.88, 124.94, 125.87, 126.11, 127.94, 128.23, 128.78, 128.99, 129.41, 129.66, 129.93, 134.81, 137.98, 149.35, 149.58, 150.53, 153.01, 157.20, 169.35. FTIR (KBr, cm^{-1}): 3056, 2581, 1760, 1632, 1584, 1514, 1479, 1433, 1388, 1345, 1197, 761, 701. HRMS (ESI): calcd for MH^+, 548.1377; found, 548.1372.

***N,N*-Bis(2-pyridylmethyl)-4-methyl-*m*-anisidine (7).** 2-Picolyl chloride hydrochloride (10.0 g, 61.0 mmol) was dissolved in 3 mL of water, and 18 mL of 5 N NaOH was added to give a pink solution. An additional 18 mL of 5 N NaOH was added to the vigorously stirred solution, after which 4-methyl-*m*-anisidine (**6**, 3.70 g, 27.0 mmol) was combined with the solution of picolyl chloride. An aliquot of cetyltrimethylammonium chloride (450 μL, 25 wt % in water) was added as a PTC, and the reaction mixture was stirred vigorously under Ar. Additional picolyl chloride hydrochloride (10.0 g, 61.0 mmol) and 5 mL of 5.0 N NaOH were added to the solution after 72 h. After 96 h, an additional portion of picolyl chloride hydrochloride (6.00 g, 36.6 mmol) was added to the solution, and after 216 h a final addition of picolyl chloride hydrochloride (7.00 g, 42.7 mmol) and 5 mL of 5 N NaOH was made. After a total reaction time of 11 days, the product was extracted into CH_2Cl_2 and dried over $MgSO_4$ to afford a dark red oil after solvent removal. Flash chromotography on basic alumina with a solvent gradient (9:1 CH_2Cl_2/EtOAc to 4:1 CH_2Cl_2/EtOAc) yielded the product as an orange oil (1.43 g, 16.6%). TLC: $R_f = 0.29$ (4:1 CH_2Cl_2/EtOAc). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.39 (3 H, s), 3.65 (3 H, s), 4.37 (4 H, s), 6.50 (1 H, dd, $J = 2.5$, 8.5 Hz), 6.61 (1 H, d, $J = 3.0$ Hz), 7.07−7.12 (3 H, m), 7.42 (2 H, d, $J = 8.0$ Hz), 7.58 (2 H, td, $J = 2.0$, 7.5 Hz), 8.52 (2 H, dt, $J = 1.0$, 5.5 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.17, 55.41, 56.70, 108.09, 108.84, 122.09, 122.36, 125.05, 131.89, 136.60, 149.26, 150.22, 158.35, 159.05. FTIR (thin film, cm^{-1}): 3392, 2940, 1608, 1589, 1501, 1433, 1163, 1045, 762. HRMS (ESI): calcd for MH^+, 320.1763; found, 320.1741.

***N,N*-Bis(2-pyridylmethyl)-3-amino-4-methylphenol (8).** A solution of *N,N*-bis(2-pyridylmethyl)-4-methyl-*m*-anisidine (**7**, 1.00 g, 3.13 mmol) in 10 mL of CH_2Cl_2 was frozen with liquid N_2, and 90 mL of 1.0 M BBr_3 (90 mmol) in CH_2Cl_2 was added via a cannula. The solution was allowed to warm slowly to room temperature and stirred under Ar for 40 h. The reaction mixture was chilled to −40 °C via a 2-propanol/dry ice bath, and MeOH was added slowly to quench the excess BBr_3. The quenched reaction mixture was diluted with ∼350 mL of water and boiled for 45 min. After the aqueous solution was cooled, neutralized to pH ∼6.5 with saturated $NaHCO_3$, and saturated with NaCl, the product was extracted into CH_2Cl_2 to give a red solid after solvent removal. Flash chromotography on basic alumina (19:1 $CHCl_3$/MeOH) yielded the product as a yellow solid (449 mg, 47.0%). TLC: $R_f = 0.26$ (19:1 $CHCl_3$/MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.38 (3 H, s), 4.33 (4 H, s), 6.49 (1 H, dd, $J = 2.5$, 7.5 Hz), 6.60 (1 H, d, $J = 2.5$), 7.04 (1 H, d, $J = 8.0$ Hz), 7.10 (2 H, dd, $J = 5.0$, 7.0 Hz), 7.45 (2 H, d, $J = 7.5$ Hz), 7.60 (2 H, td, $J = 1.5$, 7.5 Hz), 8.38 (2 H, dt, $J = 1.0$, 5.0 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.15, 59.32, 108.94, 111.42, 122.32, 122.43, 123.95, 132.38, 137.15, 148.77, 149.64, 155.87, 158.87. FTIR (thin film): 3422, 1610, 1599, 1531, 1474, 1343, 1293, 1248, 1203, 751. HRMS (ESI): calcd for MH^+, 306.1601; found, 306.1614.

9-(*o*-Carboxyphenyl)-2-chloro-6-[(2-pyridylmethyl)amino]-3-xanthanone (9). *N,N*-Bis(2-pyridylmethyl)-3-amino-4-methylphenol (**8**, 284 mg, 0.93 mmol) and 2′-carboxy-5-chloro-2,4-dihydroxybenzophenone (**4**, 272 mg, 0.93 mmol) were combined in 5 mL of methanesulfonic acid (CH_3SO_3H). The resulting dark red solution was stirred for 48 h at 70 °C. The reaction mixture was diluted with 250 mL of water, chilled to 0 °C, and slowly neutralized with saturated $NaHCO_3$. The aqueous mixture was extracted thoroughly with CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ to give a red solid after filtration and solvent removal. Flash chromatography on silica (9:1 $CHCl_3$/MeOH) yielded the product as a red solid (149 mg, 34.1%). TLC: $R_f = 0.15$ (9:1 $CHCl_3$/MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.09 (3 H, s), 4.50 (2 H, s), 6.34 (1 H, s) 6.47 (1 H, s), 6.78 (2 H, s), 7.18 (1 H, d, $J = 7.5$ Hz), 7.27 (2 H, m), 7.33 (1 H, d, $J = 7.0$ Hz), 7.64−7.72 (3 H, m), 8.10 (1 H, d, $J = 8.0$ Hz), 8.61 (1 H, d, $J = 4.5$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 48.30, 95.82, 103.59, 105.16, 111.15, 119.44, 120.97, 122.12, 123.89, 124.56, 124.94, 125.27, 126.96, 127.83, 127.91, 128.26, 129.96, 135.05, 135.72, 136.74, 148.91, 149.05, 150.13, 150.82, 158.74, 168.41. FTIR (KBr, cm^{-1}): 3402, 1643, 1595, 1560, 1494, 1434, 1329, 1287, 1016, 759. HRMS (ESI): calcd for MH^+, 471.1112; found, 471.1219.

1,4,7-Tris(benzyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (3CBZ-cyclen, 11). Cyclen (**10**, 4.35 g, 25 mmol) and Et_3N (10.5 mL) were dissolved in 250 mL of $CHCl_3$. Dibenzyl dicarbonate (20.0 g, 69.9 mmol) was dissolved in 200 mL of $CHCl_3$ and added via a syringe pump to the stirring solution of cyclen over 7 h. The reaction mixture was stirred for 24 h at room temperature. The $CHCl_3$ was removed, and flash chromatography on silica (3:17 hexanes/EtOAc) yielded the product as a white solid (10.6 g, 73.0%). TLC: $R_f = 0.35$ (EtOAc). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.91 (1 H, s), 2.76−2.87 (4 H, m), 3.29−3.77 (12 H, m), 4.88 (1 H, s), 5.05 (2 H, s), 5.14 (1 H, s), 7.18−7.34 (15 H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 45.43, 48.39, 48.69, 49.06, 49.26, 50.50, 50.95, 51.18, 66.63, 66.85, 66.97, 67.10, 127.54, 127.63, 127.74, 127.91, 128.04, 128.31, 128.37, 128.44, 136.58, 136.66, 136.88, 155.85, 156.20, 156.34. FTIR (thin film): 3213, 2940, 2827, 1696, 1609, 1498, 1419, 1365, 1259, 1159, 769, 698. HRMS (ESI): calcd for MH^+, 575.2870; found, 575.2868.

1,4,7-Tris(benzyloxycarbonyl)-10-(3-methoxyphenyl)-1,4,7,-10-tetraazacyclododecane (3CBZ-Ar-cyclen, 13). 2-Dicyclohexylphosphino-2′-(*N,N*-dimethylamino)biphenyl (450 mg, 1.14 mol), $Pd_2(dba)_3$ (200 mg, 218 μmol) and sodium *tert*-butoxide (2.6 g, 27 mmol) were combined in a Schlenk tube outfitted with a Teflon screwcap and dried in vacuo for 1 h. A 10.6 g (18.4 mmol) portion of dry **11** was transferred into the tube in 25 mL of toluene while purging with Ar. To the combined material was added 3-bromoanisidine (**12**, 2 mL, 15.8 mmol) by a syringe. After thorough purging with Ar, the tube was sealed and placed in an oil bath at 80 °C and stirred for 36 h. The reaction mixture was cooled, diluted with CH_2Cl_2, and filtered through Celite, and the solvent was removed. Flash chromatography on silica (1:1 hexanes/EtOAc) yielded the product as a white solid (3.66 g, 34%). TLC: $R_f = 0.60$ (EtOAc). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.35 (16 H, bs), 3.73 (3 H, s), 5.02 (2 H, s), 5.11 (4 H, s), 6.28 (2 H, s), 6.40 (1 H, d, $J = 7.5$ Hz), 7.10 (1 H, t, $J = 8.0$ Hz), 7.27−7.31 (15 H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 50.06, 50.54, 55.21, 67.15, 67.37, 128.08, 128.14, 128.31, 128.41, 128.60, 128.67, 130.27, 136.54, 136.76, 160.75. FTIR (thin film): 3195, 2924, 2833, 1611, 1499, 1461, 1357, 1278, 1220, 1159, 1055, 754. HRMS (ESI): calcd for MH^+, 681.3288; found, 681.3307.

1-(3-Methoxyphenyl)-1,4,7,10-tetraazacyclododecane (Ar-cyclen, 14). Pd/C (2.0 g, 10% activated) and 3CBZ-Ar-cyclen (**13**, 3.66 g, 5.38 mmol) were combined in 125 mL of MeOH and stirred under a hydrogen atmosphere (1 atm) for 24 h. The reaction mixture was filtered through Celite to give a dark yellow oil after solvent removal. Flash chromatography on silica (72:25:3 $CHCl_3$/MeOH/NH_4OH) yielded the product as a white solid (965 mg, 64.3%). TLC: $R_f = 0.24$ (97:3 $CHCl_3$/MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.52 (3 H, s), 2.63 (4 H, t, $J = 5.0$ Hz), 2.83 (4 H, t, $J = 5.0$ Hz), 2.87 (4 H, $J = 5.0$ Hz), 3.48 (4 H, t, $J = 5.0$ Hz), 3.76 (3 H, s), 6.31−6.38 (2 H, m), 6.46 (1 H, dd, $J = 2.0$, 8.0 Hz), 7.10−7.15 (1 H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 47.04, 47.10, 47.19, 52.54, 55.34, 101.99, 103.134, 129.94, 130.11, 150.90, 160.67. FTIR (thin film): 3230, 2924, 2851, 1727, 1617, 1461, 1409, 1277, 1152, 757. HRMS (ESI): calcd for MH^+, 279.2179; found, 279.2172.

1-(3-Methoxyphenyl)-7-(2-pyridylmethyl)-1,4,7,10-tetraazacyclododecane (PyAr-cyclen, 15). Ar-cyclen (**14**, 451 mg, 1.62 mmol) and 2-pyridinecarboxaldehyde (175 μL, 1.83 mmol) were combined in 50 mL of DCE and stirred. $NaBH(OAc)_3$ (515 mg, 2.43 mmol) was added, and the reaction mixture was stirred for 24 h at room temperature. Saturated $NaHCO_3$ (5 mL) was added to quench unreacted borohydride reagent. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with saturated brine, dried over $MgSO_4$, and filtered, and the solvent was removed. Flash chromatography on silica (22:2:1 $CHCl_3$/MeOH/^{i}PrNH$_2$) yielded the product as a white solid (599 mg, 63.5%). TLC: $R_f = 0.35$ (22:2:1 $CHCl_3$/MeOH/^{i}PrNH$_2$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.54−2.56 (4 H, m), 2.62−2.65 (4 H, m), 2.74 (4 H, t, $J = 5.1$ Hz), 3.37 (4 H, t, $J = 4.8$ Hz), 3.67 (2 H, s), 3.73 (3 H, s), 6.36 (1 H, dd, $J = 2.4$, 8.1 Hz), 6.50 (1 H, t, $J = 2.4$ Hz), 6.60 (1 H, dd, $J = 2.4$, 8.1 Hz), 6.93−6.98 (2 H, m), 7.10−7.24 (2 H, m), 8.24 (1 H, dd, $J = 0.6$, 3.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 47.01, 47.24, 52.45, 53.14, 55.21, 62.27, 102.27, 103.37, 109.15, 122.00, 122.87, 129.72, 136.65, 148.71, 152.03, 159.75, 160.52. FTIR (thin film): 3394, 2930, 2833, 1608, 1499, 1458, 1361, 1221, 1163, 1048, 886, 755. HRMS (ESI): calcd for MH^+, 370.2607; found, 370.2614.

1-(3-Methoxyphenyl)-4,10-(diethyl)-7-(2-pyridylmethyl)-1,4,7,-10-tetraazacyclododecane (PyEt$_2$Ar-cyclen, 16). PyEt$_2$Ar-cyclen (**15**, 675 mg, 1.83 mmol) and acetaldehyde (1 mL, mmol) were combined at 0 °C in 20 mL of DCE and stirred. The temperature of the reaction mixture was maintained between 0 and 20 °C to prevent evaporation of acetaldehyde. $NaBH(OAc)_3$ (1.63 g, 7.69 mmol) was added, and the reaction mixture was stirred for 24 h while warming to room temperature. Saturated $NaHCO_3$ (5 mL) was added to quench unreacted borohydride reagent. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with saturated brine, dried over $MgSO_4$, and filtered, and the solvent was removed. Flash chromatography on silica (22:2:1 $CHCl_3$/MeOH/^{i}PrNH$_2$) yielded the product as a white solid (412 mg, 53.0%). TLC: $R_f = 0.40$ (22:2:1 $CHCl_3$/MeOH/^{i}PrNH$_2$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.93 (6 H, t, $J = 7.2$ Hz), 2.42 (4 H, q, $J = 7.2$ Hz), 2.56−2.63 (8 H, m), 2.78 (4 H, t, $J = 5.7$ Hz), 3.55 (4 H, t, $J = 6.0$ Hz), 3.66 (2 H, s), 3.75 (3 H, s), 6.10−6.19 (2 H, m), 6.26 (1 H, d, $J = 9.6$ Hz), 7.00−7.13 (2 H, m), 7.51−7.61 (2 H, m), 8.46 (1 H, d, $J = 4.8$ Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.33, 49.37, 50.47, 51.98, 52.41, 52.79, 55.15, 61.48, 98.02, 99.86, 104.80, 121.80, 123.62, 129.71, 136.13, 148.61, 150.15, 160.07, 160.66. FTIR (thin film): 2962, 2925, 2800, 1610, 1500, 1467, 1356, 1160, 753. HRMS (ESI): calcd for MH^+, 426.3233; found, 426.3227.

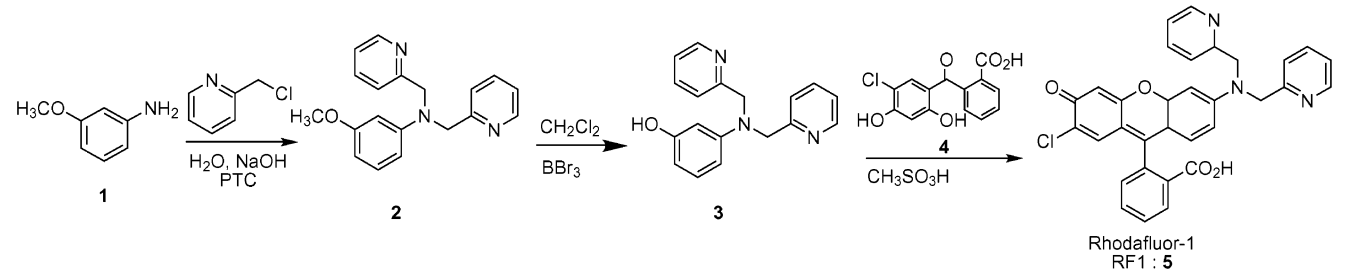
1-[9′-(*o*-Carboxyphenyl)-6′-amino-2′-chloro-3′-xanthanone]-4,10-diethyl-7-(2-pyridylmethyl)-1,4,7,10-tetraazacyclododecane (17, Rhodafluor-2, RF2). PyEt$_2$Ar-cyclen (**16**, 100 mg, 235 μmol) and 2′-carboxy-5-chloro-2,4-dihydroxybenzophenone (**4**, 275 mg, 942 μmol) were combined in 5 mL of methanesulfonic acid (CH_3SO_3H) and sealed in a thick-walled glass tube. The resulting dark red solution was stirred for 48 h at 80 °C. The reaction mixture was diluted with 100 mL of water, chilled to 0 °C, and slowly neutralized with $NaHCO_3$. The aqueous mixture was extracted thoroughly with CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ to give a red solid after filtration and solvent removal. Flash chromatography on silica (17:2:1 $CHCl_3$/MeOH/^{i}PrH$_2$N) yielded the product as a red solid (322 mg, 57.1%). TLC: $R_f = 0.41$ (17:2:1 $CHCl_3$/MeOH/^{i}PrH$_2$N). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.04 (6 H, t, $J = 6.9$ Hz), 2.84−3.18 (16 H, m), 3.72 (4 H, s), 3.83 (2 H, s), 6.44 (1 H, s), 6.77−6.79 (2 H, m), 7.18−7.22 (2 H, m), 7.31 (1 H, quad, $J = 1.2$, 5.1 Hz), 7.38 (1 H, d, $J = 7.5$ Hz), 7.63 (2 H, m, $J = 6.0$, 7.5 Hz), 7.77 (1 H, td, $J = 1.8$, 7.8), 8.10 (1 H, dd, $J = 1.5$, 7.2 Hz), 8.52 (1 H, dt, $J = 0.9$, 4.2 Hz).^{13}C NMR ($CDCl_3$, 75 MHz): δ 8.91, 21.04, 50.88, 49.722, 50.49, 52.95, 60.90, 98.90, 105.19, 113.26, 114.25, 115.39, 124.44, 125.89, 130.36, 130.63, 130.90, 130.74, 131.03, 131.45, 132.45, 134.45, 138.88, 139.06, 141.26, 150.31, 154.85, 157.34, 158.82, 159.97, 173.58, 177.64. FTIR (KBr): 3329, 2963, 2926, 1758, 1582, 1494, 1343, 1195, 1005. HRMS (ESI): calcd for MH^+, 668.2998; found, 668.2978. Anal. Calcd for $C_{39}H_{44}Cl_3N_5O_4$ (17·CH_2Cl_2): C, 62.19; H, 5.89; N, 9.30. Found: C, 62.03; H, 6.14; N, 9.27.

General Spectroscopic Methods. Ultrol grade PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) were purchased and used as received. All solutions were filtered through 0.2 μM cellulose filters before measurements. Except for the fluorescence titration experiment, Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn^{2+} stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating a 100 mM solution of 2,2′:6′,2″-terpyridine in buffered solution (50 mM PIPES, 100 mM KCl, pH 7) with aliquots of 10 mM (nominal) $ZnCl_2$ and determining the equivalence point by monitoring the absorption of the resulting complex at 321 nm ($\epsilon = 35.9 \times 10^3$ M^{-1} cm^{-1}). The Zn^{2+} stocks were prepared from 99.999% pure $ZnCl_2$. RF2 was introduced to aqueous solutions by addition of a stock solution in DMSO (1.0 mM). Graphs were manipulated and equations calculated by using Kaleidagraph 3.0. The pH values of solutions were recorded with an Orion glass electrode that was calibrated prior to each use. The experiments for measuring the pH-dependent fluorescence, quantum yield, and metal ion selectivity were performed as previously described.[5,6,8]

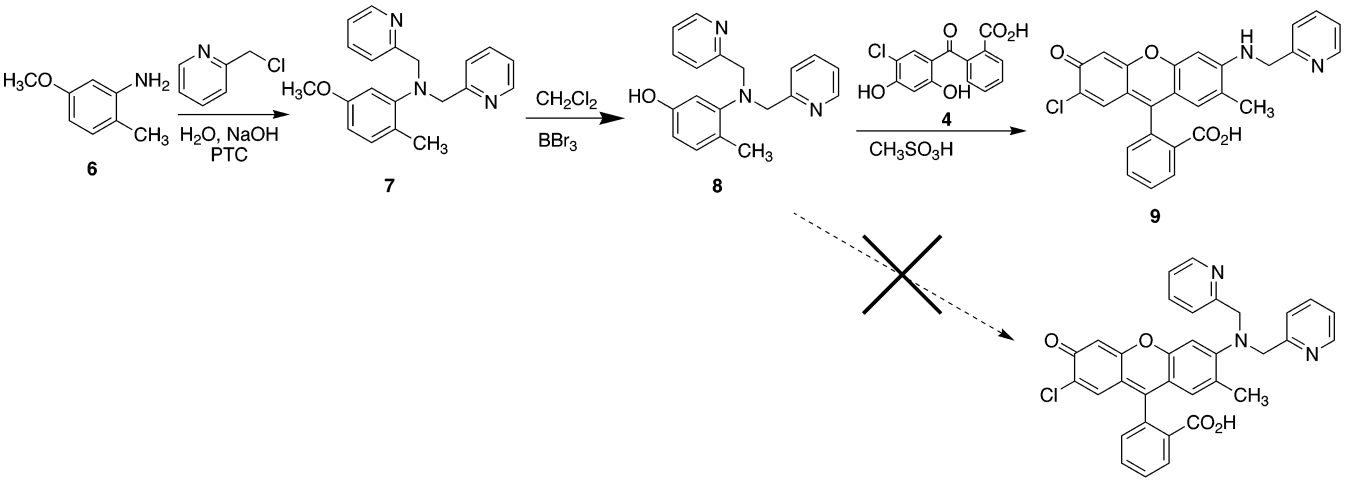
UV−Visible Spectroscopy. Absorption spectra were recorded on a Hewlett-Packard 8453A diode array spectrophotometer under the control of a Pentium II-based PC running the Windows NT ChemStation software package, or a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer-supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm path length quartz cuvettes with a volume of 1.0 or 3.5 mL.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra

Scheme 1



Scheme 2



were normalized by the manufacturer-supplied correction curves. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nm slit widths and a 240 nm/min scan speed. Fluorescence emission measurements were also acquired in a 1 cm × 1 cm quartz cell using a Spex Fluorolog-2 instrument with 1 nm bandwidth slits. All spectra were corrected for emission intensity by using the manufacturer-supplied photomultiplier curves.

Titration of Zn^{2+} Binding by Absorption and Emission Spectroscopy (K_d). A 3.0 mL solution containing 10 μM RF2 in buffer was prepared, and an initial absorbance and fluorescence measurement was made. Zn^{2+} aliquots were titrated into the solution to give final concentrations of 2.8, 5.6, 8.3, 11.1, 13.9, 16.7, 19.4, 22.2, 25.0, 27.8, 30.6, 33.3, 38.9, 44.4, 50.0, 55.6, 66.7, 77.8, 88.9, 100, 111, 139, 167, 222, 333, 444, and 556 μM, and the absorption and emission spectra were recorded. The absorption spectra were corrected for dilution, and both data sets were analyzed with SPECFIT, a nonlinear least-squares fitting program.[15] The measurements were performed in triplicate to ensure accuracy of the derived K_d value.

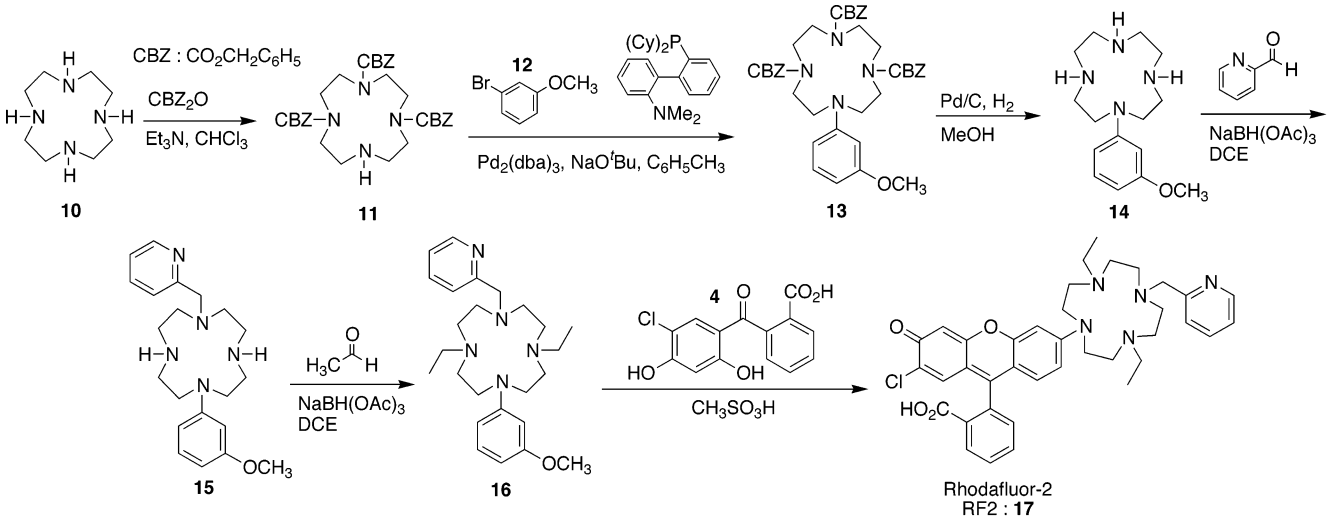
Results and Discussion

Synthesis of Rhodafluor Derivatives. Before embarking on the preparation of complex ratiometric Zn^{2+} sensors, the synthetic methods were tested by making a prototype of the RF structure (Scheme 1). Dialkylation of *m*-anisidine (**1**) with picolyl chloride affords *N*,*N*-bis(2-pyridylmethyl)-*m*-anisidine (**2**) in fair yield. Subsequent removal of the methyl protecting group with boron tribromide (BBr_3) provides *N*,*N*-bis(2-pyridylmethyl)-3-aminophenol (**3**), one of the required fragments for the synthesis of RF1 (**5**). A variety of reagents were screened for catalyzing the condensation of **3** with 2′-carboxy-5-chloro-2,4-dihydroxybenzophenone (**4**) to yield RF1. Unlike the synthesis of the structurally related Ca^{2+} sensor,[13] reactions catalyzed by $ZnCl_2$ in THF (200 °C, sealed vessel) failed to yield the desired product. Presumably zinc binding to **3** inhibits the reaction. RF1 was isolated successfully from the reaction of **3** and **4** in neat methanesulfonic acid (CH_3SO_3H).

The affinity of RF1 for Zn^{2+} is predicted to be weak since it contains only poor metal-binding units. RF1 has an aniline nitrogen atom with a partial positive charge at neutral pH and two pyridine donors. A practical sensor requires additional metal binding ligands to enhance binding affinity. One approach to synthesizing more elaborate targets involves functionalization of the parent RF1 skeleton. The presence of a methyl group adjacent to the di(2-picolyl)amine (DPA) provides potential for modification of the chemical structure using the methods devised for the synthesis of ZP2 and ZP4.[5,6] Following a parallel synthetic scheme to that employed for RF1, the *N*,*N*-bis(2-pyridylmethyl)-3-amino-4-methylphenol (**8**) analogue, which would afford the desired compound, was prepared in gram quantities. Reaction of **8** with **4** in CH_3SO_3H failed to produce the desired compound, however (Scheme 2). An unexpected decomposition pathway results in the elimination of one of the 2-picolyl groups from the aniline nitrogen. Elaboration of **9** proves to be impractical because aniline nitrogen atoms are not particularly reactive, and installation of protecting groups on the free phenol is not trivial with these hybrid fluorophores. In addition, the aromatic ring system of RF1 seems inert to electrophilic

(15) Binstead, R.; Zuberbühler, A. D.; Jung, B. *SPECFIT*, 3.0.16 ed.; Spectrum Software Associates: Chapel Hill, NC, 2000.

Scheme 3



aromatic substitution reactions like the Mannich reaction used to prepare ZP1.[5]

Manipulation of an assembled RF compound presents several synthetic difficulties. Alternatively, the preparation of 3-aminophenols is straightforward and functionalization of the Zn^{2+}-binding ligand prior to formation of the RF framework is an appealing strategy. Investigations into macrocyclic polyamines indicate that these ligands act as selective, high-affinity receptors for Zn^{2+}.[16] Recent progress in Pd-catalyzed amination chemistry demonstrates that macrocyclic amines can be coupled with aromatic bromides,[14,17] thereby avoiding elaborate multistep synthesis required by more conventional techniques.[18]

The synthesis of a cyclen-based RF target is outlined in Scheme 3. Installation of protecting groups on three nitrogen atoms restricts the Pd coupling to a single site on the cyclen ring. The preparation of the triprotected compound from commercially available cyclen (**11**) was accomplished by using procedures for the synthesis of analogous Boc-protected cyclen.[19] Coupling of the 3-bromoanisidine (**12**) with the 3CBZ-cyclen (**11**) proceeds in reasonable yield and provides multigram quantities of the desired product. The efficient synthesis of **11** represents a significant advance in RF synthesis. Removal of the CBZ protecting groups occurs under mild conditions in high yield to afford Ar-cyclen (**14**). One commonly encountered feature of Zn^{2+} cyclen complexes is the presence of an additional pendant chelating ligand.[20] Reaction of **14** with 2-pyridinecarboxaldehyde under reducing conditions yields the 1,7-substituted PyAr-cyclen (**15**) with excellent selectivity. None of the 1,4-product is observed, and only a small amount of the compound containing a second pyridyl arm is recovered when the quantity of aldehyde is restricted to 1 equiv. When submitted to identical reductive amination conditions using an excess of acetaldehyde, the remaining two secondary nitrogen atoms of **15** undergo alkylation yielding the $PyEt_2Ar$-cyclen (**16**).

Methyl ether protecting groups are robust, and deprotection often requires harsh conditions. Only limited quantities of the corresponding 3-aminophenol could be recovered following removal of the methyl group of **16** with BBr_3. Test reactions substituting the methyl ether **2** for the phenol **3** in the synthesis of RF1 indicated that removal of the ether was unnecessary for assembly of the final compound. Avoiding a deprotection step eliminates a low-yielding step in the synthesis. Reaction of **16** with **4** in neat CH_3SO_3H provides RF2 (**17**) in high yield. RF2 is prepared in 3.1% overall yield from cyclen in six steps.

Fluorescence Properties of RF2. The absorbance maximum of RF2 under simulated physiological conditions (50 mM PIPES, 100 mM KCl, pH 7) occurs at 514 nm ($\epsilon = 82.7 \times 10^3\ M^{-1}\ cm^{-1}$) with an emission maximum at 539 nm. The quantum yield of the RF2 in the presence of EDTA to scavenge adventitious metal ions is 0.36. In the presence of Zn^{2+}, the absorbance maximum shifts slightly to 510 nm ($\epsilon = 50.8 \times 10^3\ M^{-1}\ cm^{-1}$), the maximum emission remains at 539 nm, and the quantum yield increases to 0.56. The two limiting resonance forms of the RF fluorophore are the iminophenoxy mesomer, which has rhodamine-like fluorescence properties, and the aminoquinone mesomer, which has optical properties characteristic of fluorescein (Figure 1). A

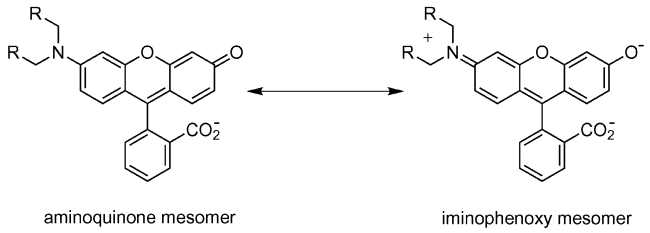
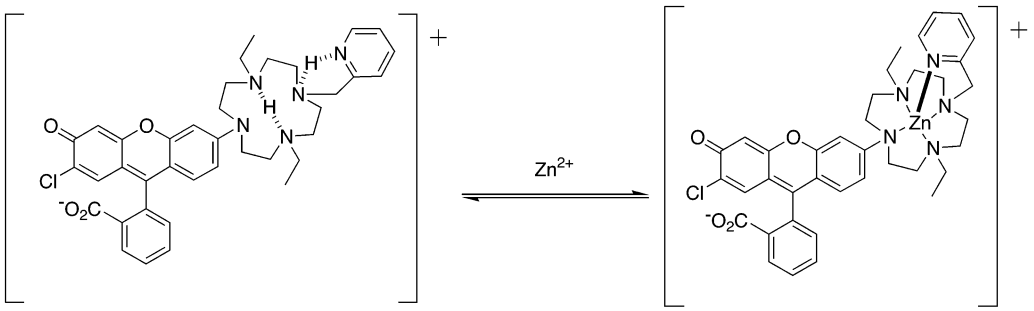


Figure 1. The two limiting resonance forms of the rhodafluor fluorophore. The aminoquinone mesomer has fluorescence properties characteristic of fluorescein, and the iminophenoxy has rhodamine-like fluorescence properties.

(16) Kimura, E. *Prog. Inorg. Chem.* **1994**, *41*, 443−491.
(17) Beletskaya, I. P.; Averin, A. D.; Bessmertnykh, A. G.; Denat, F.; Guilard, R. *Tetrahedron Lett.* **2002**, *43*, 1193−1196.
(18) Hirano, T.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *Angew. Chem., Int. Ed.* **2000**, *39*, 1052−1058.
(19) Kimura, E.; Aoki, S.; Koike, T.; Shiro, M. *J. Am. Chem. Soc.* **1997**, *119*, 3068−3076.
(20) Koike, T.; Watanabe, T.; Aoki, S.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1996**, *118*, 12696−12703.

Scheme 4



change in the contribution from these two limiting resonance forms to the electronic structure of the fluorophore in the complexed and uncomplexed states is required to alter significantly the optical properties of the sensor. The small shift in the absorbance spectrum, and the fluorescein-like wavelengths observed in the presence and absence of Zn^{2+}, suggest that RF2 begins and remains in the aminoquinone form during metal ion binding.

The negligible Zn^{2+}-induced spectral shifts prompted an examination of the pH dependent absorption and emission wavelengths of RF2. Under basic conditions (pH 12, 100 mM KCl), the absorption maximum occurs at 526 nm with an emission maximum at 539 nm. Under acidic conditions (pH 5, 100 mM KCl), the absorption maximum occurs at 507 nm with an emission maximum at 529 nm. The fluorescence intensity of RF2 is dependent on protons. Fitting of the integrated emission intensity reveals pK_a values at 11.5 and 6.4, corresponding to increases in fluorescence, and one value at 2.9 as fluorescence decreases (Figure 2). Diminished fluorescence under acidic conditions is associated with the formation of a nonfluorescent isomer of RF2 similar to the one adopted by ZP sensors at low pH.[5,6] The pK_a values at 11.5 and 6.4 indicate protonation of amine atoms in the cyclen macrocycle. When deprotonated, the free electrons of amine atoms participate in quenching of the fluorophore excited state by a PET mechanism. This proposed quenching mechanism is consistent with the observed increase in the quantum yield upon Zn^{2+}-binding. The pH dependence of RF2 absorption and emission also provides an explanation for the minimal Zn^{2+}-induced wavelength shifts. Protonation of the macrocycle at neutral pH forces RF2 to adopt the aminoquinone form in the both the Zn^{2+}-free and complexed forms, limiting the contribution of the iminophenoxy mesomer to the fluorescence properties (Scheme 4).

The fluorescence increase and binding affinity of RF2 for Zn^{2+} were characterized by titration of the sensor with μM

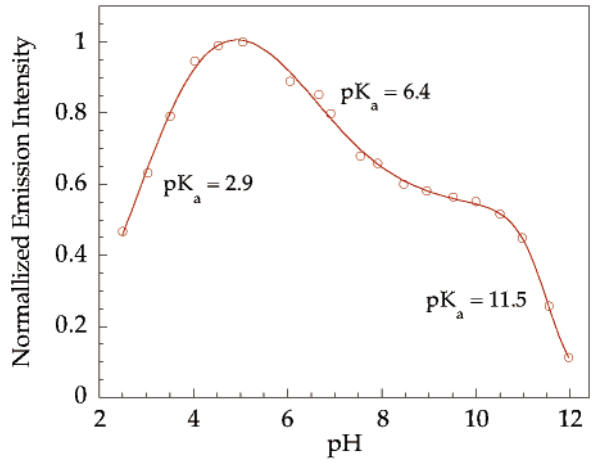


Figure 2. Plot of the normalized integrated emission intensity versus pH for RF2. The increase in fluorescence corresponds to protonation of the amine atoms in the cyclen macrocycle, and the decrease corresponds to the formation of a nonfluorescent isomer.

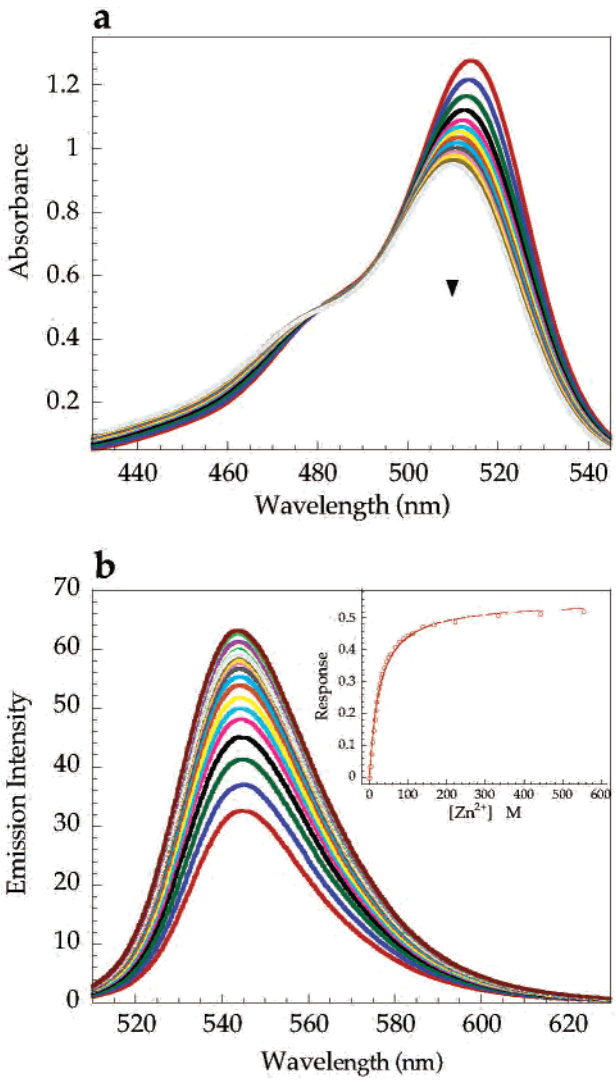


Figure 3. The absorption (a) and emission (b) spectra of RF2 after addition of Zn^{2+} to give final concentrations of 5.6, 11.1, 16.7, 22.2, 25.0, 27.8, 33.3, 44.4, 55.6, 66.7, 77.8, 88.9, 100, 111, 139, 167, 222, 333, 444, and 556 μM. Emission increases at 539 nm while absorption decreases at 514 nm over the course of the titration.

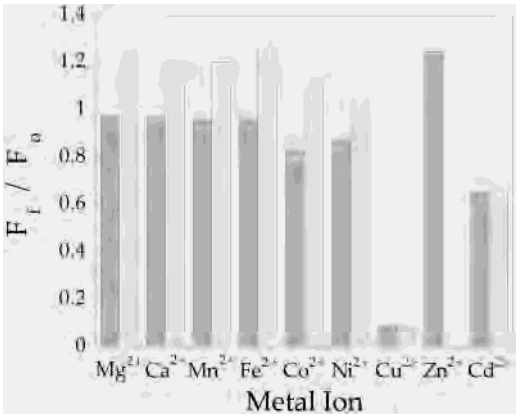


Figure 4. Fluorescence response of RF2 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Initial spectra were acquired in 100 mM KCl, 50 mM PIPES, 10 μM EDTA pH 7.00 at 25 °C. Excitation was provided at 514 nm, and the emission was integrated between 490 and 625 nm. Dark bars: Aliquots of concentrated stock solutions (10 mM) of each metal ion were added to the solution to provide 50 μM total metal ion, and the fluorescence response was calculated. Light bars: Zn^{2+} (50 μM) was added subsequently to the solution containing the metal ion, and the response was measured.

concentrations of metal ion and measurement of the absorption and emission spectra (Figure 3). The absorption changes fit a K_d of 13.5 ± 0.5 μM. The affinity of RF2 for Zn^{2+} is lower than for some cyclen complexes,[20] but comparable in value to compounds that contain an aniline nitrogen atom in the macrocycle.[18] With the exception of Cu^{2+} and Cd^{2+}, which bind to RF2 and quench the fluorescence, other divalent transition metals induce only slight quenching in the fluorescence intensity (Figure 4). Binding by these metal ions appears to be weak since subsequent addition of Zn^{2+} to these RF2 solutions enhances fluorescence.

Conclusions

We have prepared a first generation ratiometric sensor for Zn^{2+} exploiting organometallic methodology for coupling aryl bromides with azamacrocycles. This approach provides a straightforward and convenient means to access a compound that otherwise would require a difficult, multistep route using conventional organic techniques. In addition to synthetic ease, this method permits assembly of the Zn^{2+} binding ligand prior to formation of the fluorophore skeleton, avoiding manipulation of a more sensitive compound.

Since the amine atoms of RF2 are susceptible to protonation, the sensor prefers the aminoquinone mesomer in the presence and absence of Zn^{2+} at physiological pH. The insignificant contribution from the iminophenoxy resonance form in the uncomplexed state prevents RF2 from exhibiting the desired shifts in the excitation and emission wavelength upon Zn^{2+} binding. RF2 does act as a modest PET sensor, however, with an ~50% enhancement in quantum yield upon Zn^{2+} binding. This property renders RF2 of immediate value for application in situations where our previous tight binding (<1 nM) sensors are inadequate. Variation of the metal ion receptor should allow attenuation of the properties of future RF sensors to achieve the desired wavelength changes. Application of the methodologies described here will allow these changes to be made in a simple and efficient manner.

Acknowledgment. This work was supported at MIT by the McKnight Foundation for the Neurosciences and Grant GM65519 from the National Institute of General Medical Sciences. We thank X.-X. Zhang for his guidance and helpful suggestions in running the Pd-catalyzed coupling reactions, and Matt Clark for valuable discussions.

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