Chelation-Enhanced Fluorescence of Anthrylazamacrocycle Conjugate Probes in Aqueous Solution[1]

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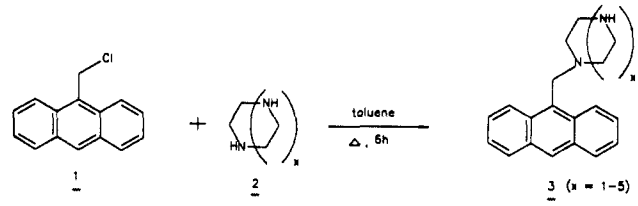
Abstract: In this paper, we report the synthesis of several anthrylazamacrocycles designed to provide chelation-enhanced fluorescence on complexation of nonquenching metal ions [e.g., Zn(II) and Cd(II)] in totally aqueous solution. The N_3, N_4, N_5, and N_6 derivatives are water-soluble compounds whose fluorescence is enhanced by addition of Zn(II) and Cd(II) and quenched by addition of Cu(II) and Hg(II). While the background fluorescence of anthrylazamacrocycles is higher in water than in aprotic solvents, large (20–190-fold) overall fluorescence changes are still obtained on complexation. The titration of these metal ions is unaffected at pH 12 by even very high (1 M) concentrations of nonbinding metals such as Na^+. pH–fluorescence profiles for the anthrylazamacrocycles support intramolecular amine fluorescence quenching at basic pH. These results suggest that the large body of literature on the synthesis of selective azamacrocycle binders can be used in a direct way for the design of conjugate fluorescent probes for those metals.

Fluorometric methods have proven useful for the assay of metal ions in solution.[3] Recently, in vivo studies of calcium-selective fluorescent probes have been reported by Tsien.[4] Most such analytical methods reported to date involve complexation of metal ions with aromatic heterocyclic ligands ("intrinsic" fluoroionophores). In 1977, Sousa described the synthesis of naphthalene–crown ether probes in which the fluorophore π-system is insulated from the donor atoms by at least one methylene group ("conjugate" fluoroionophores).[5a] These compounds demonstrate fluorescence changes of up to 6-fold upon the binding of alkali-metal salts in 95% ethanol glass at 77 K; the observed changes were attributed to a heavy atom effect (for Cs^+ and Rb^+) and/or a complexation-induced change in triplet energy relative to ground and excited singlet state energies. Subsequent reports by Bouas-Laurent and Lehn, Wolfbeis, de Silva, Street, Sousa, and our group have built on this original premise, in which binding of metals to crowns and azacrowns has been coupled to emission changes of covalently attached fluorophores.[5b-j] While such conjugate probe methods offer considerable potential and flexibility in the design of metal-selective fluoroionophores, large fluorescence changes to date have been seen only in nonaqueous solution. In at least one example, even a small amount of water decreases the sensitivity significantly;[5e] our own work with 9,10-bis(TMEDA)anthracene afforded chelation-enhanced fluorescence (CHEF) in acetonitrile but not in water.[5h] Of course, assays in totally aqueous media are better suited to many potential applications. Accordingly, we now report large (25–190-fold) CHEF effects in totally aqueous environment using anthrylazamacrocycle conjugate probes.

Scheme I



a, $x = 1$; **b**, $x = 2$; **c**, $x = 3$; **d**, $x = 4$; **e**, $x = 5$

Synthesis

Anthrylazamacrocycles **3a–e** were synthesized (Scheme I) by the reaction of 9-(chloromethyl)anthracene (**1**) with an excess of the appropriate azamacrocycle (**2a–e**) by using an adaptation of a literature procedure.[6] The synthesis of **3e** could not be effectively carried out in toluene solution, but reaction in acetonitrile worked well. Attempted purification by chromatography on neutral alumina led to greatly lower yield of product; compound **3e** did not elute from an alumina column at all. In addition, attempted purification by ion-exchange chromatography[7] led to decomposition.

The free bases of **3a–e** were obtained as oils after selective basic extraction. All compounds were subsequently isolated as HCl salts by precipitation from ethanol with concentrated HCl; attempts at crystallization of HCl salts did not afford purification. Characterization of each compound was accomplished by using ^1H NMR, ^{13}C NMR, and FAB mass spectrometry, high-resolution FAB mass spectrometry, and UV. In samples of **3d** and **3e**, a small (<5%) impurity of the starting azamacrocycle could be observed in the ^1H NMR spectrum and quantitated by integration; as we were unable to purify these compounds by chromatography or crystallization, fluorescence experiments were performed using these samples. The elemental composition of these samples was shown by microanalysis to include varying amounts of H_2O, as expected for moderately hygroscopic polyammonium salts. However, establishment of purity by microanalysis has not been conclusive (see Experimental Section). We have demonstrated

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(2) Recipient of Ohio State University and Amoco graduate fellowships.

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(7) An Abridged Outline Dealing with the Practical Aspects of Ion Exchange Chromatography. Technical Bulletin, Ace Glass Co., Vineland, NJ.

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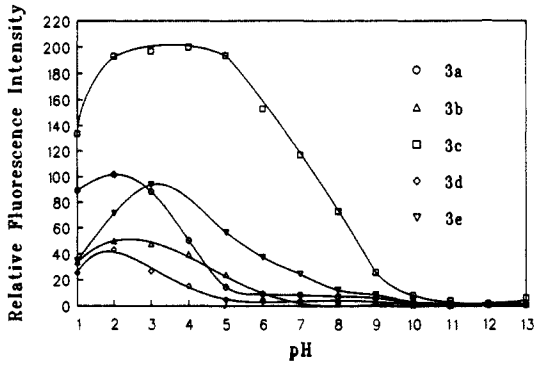

Figure 1. pH–fluorescence profiles for 10 μM solutions of anthrylazamacrocycles **3a** (○), **3b** (△), **3c** (□), **3d** (◇), and **3e** (▽). Excitation was at 335 ± 3 nm; emission was measured at the emission maximum centered near 416 nm. pH's were maintained by using the following solutions (all 0.1 M): trichloroacetate (pH 1), dichloroacetate (pH 2), chloroacetate (pH 3), acetate (pH 4 and 5), MES (pH 6), HEPES (pH 7 and 8), CHES (pH 9), CAPS (pH 10 and 11), tetrabutylammonium hydroxide (pH 12), NaOH (pH 13).

Table I. Relative Fluorescence Intensities of (9-Anthrylmethyl)azamacrocycles in the Presence of Various Metal Ions[a]

metal ion	3a	3b	3c	3d	3e
	2.5	1.3	9.0	2.3	3.5
Ca(II)	2.5	1.8	9.4	2.2	4.1
Al(III)	2.5	1.4	9.8	1.9	3.3
Pb(II)	4.0	1.8	4.1	2.6	2.7
Cu(II)	2.5	0.6	1.6	1.6	1.9
Cd(II)	2.7	13.8	81.0	20.3	107.1
Zn(II)	2.6	44.2	129.2	17.8	138.7

[a] All solutions were buffered with 0.1 M CAPS buffer at pH 10.0 and were 10 μM in anthrylazamacrocycle and 20 μM in the appropriate metal perchlorate. Excitation was at 335 ± 3 nm; emission was measured at the emission maximum centered near 416 nm. The apparent small CHEF seen with Pb(II) and compound **3a** is instead likely due to scattering by precipitated metal ion, seen only in this case because **3a** does not complex Pb(II).

homogeneity using ^{13}C NMR, which clearly reveals a single anthrylazamacrocycle in each sample. Integration of the ^1H NMR spectrum in each case demonstrated a 1:1 ratio of anthracene to azamacrocycle protons, confirming that overalkylation had not occurred.

Results

The pH–fluorescence profiles for compounds **3a–e** are shown in Figure 1. While fluorescence quenching is expected at high pH, we also observe some form of quenching under strongly acidic conditions; such a decrease has been observed with other fluorophores.[8] This fluorescence decrease cannot be due simply to hydrolysis of anthrylazamacrocycles to anthrylmethanol and the azamacrocycle, because anthrylmethanol is fluorescent and sufficiently soluble under these conditions to remain in solution. Protonation of the anthracene ring may contribute to this effect; the pK_a of ground-state anthracene is 3.3.[9] In addition, we observe an irreversible, acid-catalyzed, photochemically induced reaction of these anthrylazamacrocycles at acidic pH's. A 10 μM solution of **3c** in 0.1 N DCl/D_2O gave the expected ^1H NMR spectrum, and storage in the dark overnight revealed only a small amount of additional cyclen indicating hydrolysis. However, subsequent exposure of the NMR sample to long-wavelength light from a hand-held UV source for 20 min resulted in rapid formation of a precipitate and loss of **3c** as determined by NMR. Characterization of the decompostion products has not been successful to date. Given the strong basicity of excited-state anthracene

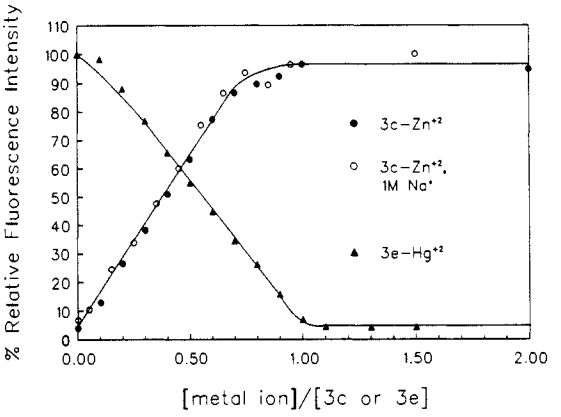

Figure 2. Titration of the fluorescence of anthrylazamacrocycles **3c** (10 μM) and **3e** (100 μM) by added metal ions. The three experiments shown are as follows: titration of **3c** in pH 12 buffer with $Zn(ClO_4)_2$ (●); titration of **3c** in pH 12 buffer with $Zn(ClO_4)_2$ and 1 M $NaClO_4$ (○); titration of **3e** in pH 6 buffer with $Hg(ClO_4)_2$ (▲). The CHEQ of **3e** by Hg(II) ion was accomplished at pH 6 because, while the azamacrocycle is sufficiently deprotonated at this pH to bind Hg(II), it is also sufficiently protonated to display some background fluorescence (Figure 1). Excitation was at 335 (**3c**) or 310 (**3e**) nm; emission was measured at the emission maximum centered near 416 nm.

[$pK_a(S_1)$ = 17.0],[9] a reaction pathway involving the anthracenium ion seems likely.

Fluorescence changes upon the addition of various metals are shown in Table I, and titrations with Zn(II) and Hg(II) are shown in Figure 2. Both chelation-enhanced fluorescence (CHEF) and chelation-enhanced quenching (CHEQ) are observed for these ions, with overall emission changes of 25-fold and 20-fold, respectively.[10] At pH 12, the presence of a large (100 000-fold) excess of added $NaClO_4$ does not interfere with the titration of Zn(II) ion. The absorption spectra remain unchanged in the Zn(II) titration experiments. In titrations with Cu(II) (not shown) a new absorption [λ_{max} 294 nm; assigned to the Cu(II)–azamacrocycle complex] grew in that tailed significantly into the anthracene absorption region (330–400 nm). In an effort to test the limits of signal range in aqueous solution, we carried out the titration of **3c** with Cd(II) under strongly basic conditions (pH 13); because of the very low background fluorescence at this pH, a CHEF effect of 190-fold was obtained upon addition of a saturating amount (2 equiv) of $Cd(ClO_4)_2$.

Discussion

Fluorescence as a Function of pH. An observed fluorescence dependence on pH is in keeping with the intramolecular amine quenching mechanism that has been described previously.[5c,d,h,11] Protonation of an amine group in fluorophore–amine conjugates results in the elimination of photoinduced electron transfer. Therefore, fluorescence is expected to be a function of pH, and pH measurement using anthrylamines has been described by de Silva.[12] The maximal emission intensity of equimolar solutions of **3a–e** at pH 2–3 varies significantly, with **3c** clearly the most fluorescent; this interesting but unexplained finding is the focus of current study. In addition, it is noteworthy that compound **3c** functions as a fluorescent pH indicator with a nearly linear response between pH 5 and 10, although its metal-binding properties make **3c** nonideal for this purpose.

Our data indicate that the deprotonation (either full or fractional) of a single, specific ammonium ion in these structures likely

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(9) Ireland, J. F.; Wyatt, P. A. H. *Adv. Phys. Org. Chem.* **1976**, *12*, 131 (see Table 4).

(10) While we are not equipped to measure absolute quantum efficiencies, the emission intensity of the **3c**–Zn(II) complex was 30% that of an equimolar solution of 9-methylanthracene in ethanol solution with a known quantum yield of 0.33 (Parker, C. A.; Joyce, T. A. *J. Chem. Soc., Chem. Commun.* **1967**, 744). This imperfect comparison allows at least a crude estimation of 0.1 for the quantum efficiency of the **3c**-Zn(II) complex.

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accounts for a majority of the observed intramolecular quenching. The fluorescence of **3a** reaches 92% of its minimum value at pH 6, which is related to the first deprotonation of the piperazinium dication [$pK_a(1) = 5.6$]. Similarly, the fluorescence intensity for tetraamine **3c** reaches 97% of its minimum value at pH 10 and the parent azacrown **2c** has $pK_a(3) = 9.7$.[13] We postulate that protonation at the benzylic nitrogen in all five anthrylazamacrocycles is the key step leading to large fluorescence enhancements; this idea is supported by the studies of Thomas[14] and of Davidson[15] on the distance dependence of exciplex formation in a series of homologous naphthyl- and anthrylalkylamines.

Fluorescence as a Function of Added Metal Ion. Our survey of fluorescence changes involving various anthrylazamacrocycles and metal ions has permitted us to put potential interactions into three catagories:

Case 1: Complexation with Fluorescence Enhancement. If the metal ion itself is not quenching,[17] binds to the azamacrocycle,[18] and does not form a complex capable of absorbing the emitted light, a large CHEF effect (25–190-fold, depending on the metal, the anthrylazamacrocycle, and the pH used) is observed. Zn(II) and Cd(II) display this type of behavior (Table I). A Zn(II) titration of **3c** demonstrates a linear response, as shown in Figure 2; nearly complete complexation is observed on addition of 1 equiv of Zn(II) to a 10 μM solution of **3c**.

The CHEF effects we observe on the binding of Zn(II) and Cd(II) are significantly smaller than the >1000-fold enhancement we have previously reported in acetonitrile solution.[5h] We have observed that the background fluorescence of noncomplexed anthrylpolyamines increases as the hydrogen-bonding capability of the solvent increases. For example, the background fluorescence of a 10 μM solution of anthrylazamacrocycle **3c** in pH 12 buffer is 230% that of an equimolar solution of the free base of **3c** in acetonitrile. This sensitivity to protic solvent is quite predictable; hydrogen bonding of the benzylic nitrogen will also serve to remove electron density from that position and reduce electron-transfer quenching. Thus, fluorescence becomes a potentially useful tool for studying the microenvironment about the benzylic nitrogen.

Case 2: Complexation with Intracomplex Quenching. If a quenching metal ion (e.g., open-shell, paramagnetic, large or easily reducible cation[19]) binds tightly to the anthrylazamacrocycle derivative, intracomplex quenching takes place. The fluorescence of compounds **3b–e** can be titrated down as shown in Figure 2 for **3e** and Hg(II). This CHEQ experiment was carried out at [**3e**] = 100 μM. The experiment also gives a linear response when carried out at [**3e**] = 10 μM, but the very small signal obtained near the end of this titration could not be accurately distinguished from zero fluorescence, making the determination of an end point at a 1:1 stoichiometry impossible. At the concentrations used in this latter study (10 μM in anthracene derivatives and 20 μM in metal ions), we do not see any intermolecular quenching of **3a** fluorescence by the metal ions used.

Case 3: No Complexation. If the binding interaction is not strong enough there is no effect on the fluorescence. As shown in Table I, the fluorescence intensity of solutions of piperazinyl derivative **3a** does not change on addition of metal ions. Ca(II) and Al(III), both very weak binders to the macrocyclic polyamines,[20] have no effect on the fluorescence of any anthrylazamacrocycle. Na^+ also has no effect, and as shown in Figure 2, the titration profile of **3c** with Zn(II) is virtually unaffected even by the presence of a 100000-fold excess of Na^+ at pH 12.

Summary

In summary, we have shown for the first time that conjugate fluorescent probes, fluoroionophores in which the metal ligand is not an integral part of the aromatic π-system, demonstrate large (20–190-fold) signal changes on transition metal ion binding in 100% aqueous solution. The pH dependence of fluorescence emission intensity is consistent with the elimination of photoinduced electron transfer via amine protonation, and it seems likely that CHEF effects on the binding of Zn(II) and Cd(II) result from the same mechanism. Under some conditions, the presence of 1 M sodium ion does not interfere with the titration of micromolar amounts of Zn(II). The binding of inherently quenching metals, such as Cu(II) and Hg(II), results in intracomplex quenching even though both ions are also expected to complex to the quenching amine. In that only a benzylic amine results in a nonemissive exciplex, it seems likely that it is principally the benzylic amine that is responsible for quenching of the uncomplexed anthrylazamacrocycles. Hydrogen bonding to the benzylic amine also results in increased fluorescence, although to a lesser extent than protonation or metal ion complexation. The use of anthrylazamacrocycles as conjugate probes is therefore facilitated by their ease of synthesis, but suffers from an inability to purify them by standard recrystallization or chromatographic methods. However, the titration data shown in Figure 2 ensure that CHEF and CHEQ effects are not due to interaction with a small amount of a highly fluorescent impurity.

Experimental Section

General Procedures. Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Microanalyses were carried out at Canadian Microanalytical Service, New Westminster, BC. Mass spectra were obtained by use of a Kratos-30 mass spectrometer. FT-NMR spectra were obtained at 11.75 (500 MHz) or 7.0 T (300 MHz). UV spectra were obtained on a Hewlett-Packard 8451A diode array spectrophotometer in pH 7.0 HEPES buffer (0.1 M); all wavelength data reported are ±1 nm. Fluorescence measurements were made on a Perkin-Elmer LS-5 luminescence spectrometer; both emission and excitation slit widths were 3 nm. 9-(Chloromethyl)anthracene (**1**), piperazine (**2a**), 1,4,7-triazacyclononane (**2b**), and 1,4,7,10,13,16-hexaazacyclooctadecane (**2c**) trisulfate were purchased from Aldrich Chemical Co., Milwaukee, WI. 1,4,7,10-Tetraazacyclododecane (**2d**; "cyclen") tetrahydrochloride and 1,4,7,10,13-pentaazacyclopentadecane (**2e**) pentahydrochloride were purchased from Parish Chemical Co., Orem, UT. All metal perchlorates were obtained from GFS Chemical, Columbus, OH.

The free bases of cyclic polyamines were generated from salts by dissolving 1 g of the salt in 6 M aqueous NaOH solution (20–125 mL) with sonication, followed by extraction with $CHCl_3$ (5 × 30 mL). The organic layer was dried over Na_2CO_3 and evaporated to dryness, leaving the free base as a colorless solid.

The use of microanalysis for establishing sample homogeneity has not been satisfying. Determinations of the carbon/chlorine ratio, which should be independent of the amount of water present, often revealed fractional amounts of HCl in the solid samples; in addition, nitrogen analyses were consistently low for the N5 and N6 compounds. As a test of the microanalytical method, we prepared the HCl salt of *N,N'*-bis(3-aminopropyl)ethylenediamine; microanalysis again revealed a fractional amount of HCl, and the analysis could not be reconciled with the theoretical to within 0.4% even upon addition of H_2O. However, both ^{13}C and ^1H NMR indicate the presence of a single anthracene compound in each sample. In our experience, the UV extinction coefficients for anthrylpolyamines vary with structure, even for microanalytically pure samples, and are therefore useful only for crude quantitation of sample purity. Consequently, microanalytical data are not reported for samples that could not be analyzed in this way. Solution concentrations were determined by using the carbon analysis for each sample; the ^{13}C NMR of each sample demonstrated no carbon-based impurities (other than the small, quantifiable amount of free azamacrocycle in samples of **3d** and **3e**).

9-(1',4'-Diazacyclohexyl)methylanthracene (3a). To a solution of piperazine (**2a**; 0.43 g, 5.0 mmol) in toluene (10 mL) was added 9-

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(14) Chandross, E. A.; Thomas, H. T. *Chem. Phys. Lett.* **1971**, *9*, 393.
(15) Brimage, D. R. G.; Davidson, R. S. *J. Chem. Soc., Chem. Commun.* **1971**, 1385.
(16) A standardized solution of $Hg(NO_3)_2$ was obtained from Aldrich Chemical Co., Milwaukee, WI (Catalog No. 20,729-2), which gave results identical with those obtained with solutions we prepared with reagent-grade $Hg(ClO_4)_2$.
(17) Masuhara, H.; Shioyama, H.; Saito, T.; Hamada, K.; Yasoshima, S.; Mataga, N. *J. Phys. Chem.* **1984**, *88*, 5868.
(18) For binding constants of various metal ions to parent azacrowns, see: (a) Kodama, M.; Kimura, E.; Yamaguchi, S. *J. Chem. Soc., Dalton Trans.* **1980**, 2536. (b) Kodama, M.; Kimura, E. *J. Chem. Soc., Dalton Trans.* **1978**, 1081.
(19) (a) Varnes, A. W.; Dodson, R. B.; Wehry, E. L. *J. Am. Chem. Soc.* **1972**, *94*, 946. (b) Kelmo, J. A.; Shepherd, T. M. *Chem. Phys. Lett.* **1977**, *47*, 158. (c) Formosinho, S. *J. Mol. Photochem.* **1976**, *7*, 13.
(20) For a review, see: Poonia, N. S.; Bajaj, A. V. *Chem. Rev.* **1979**, *79*, 389.

(chloromethyl)anthracene (**1**; 0.23 g, 1.0 mmol). The resulting solution was heated under reflux for 6 h. After cooling, the precipitated salts were removed by filtration. The resulting clear solution was washed with 5% aqueous NaOH solution (2 × 10 mL) and with H_2O (4 × 5 mL), and then the organic layer was dried over Na_2SO_4. After removal of the solvent in vacuo, the residue was dissolved in ethanol (10 mL) and concentrated HCl (1.5 mL) was added. On cooling, the hydrochloride salt precipitated and was collected by filtration and dried in vacuo at 80 °C to afford **3a** (0.16 g, 58%): mp 265 °C dec (darkens above 200 °C); UV (λ_{max}[pH 7]) 368 nm (ϵ 11 700 M^{-1} cm^{-1}); ^1H NMR ($CDCl_3$, free amine form) δ 2.58 (m, 4, CH_2CH_2), 2.83 (m, 4, CH_2CH_2), 4.43 (s, 2, $ArCH_2$), 7.49 (m, 4, ArH), 8.01 (d, 2, ArH), 8.41 (s, 1, ArH), 8.52 (d, 2, ArH); ^{13}C NMR (D_2O) δ 42.39, 50.08, 53.76, 124.30, 126.48, 128.51, 130.38, 131.29, 131.94, 132.08;[21] FAB mass spectrum, *m/e* 276 (M^+); high-resolution FAB mass spectrum of free base calcd for $C_{19}H_{20}N_2$ 276.163, measd 276.164; Anal. for $C_{19}H_{20}N_2 \cdot 2HCl \cdot H_2O$: C, H, Cl, N.

9-(1′,4′,7′-Triazacyclononyl)methylanthracene (3b). 1,4,7-Triazacyclononane (**2b**; 0.64 g, 5 mmol) and 9-(chloromethyl)anthracene (0.23 g, 1 mmol) were reacted as described for **3a** to afford anthrylazamacrocycle **3b** (0.18 g, 56%): mp 177 °C dec; UV (λ_{max}[pH 7]) 370 nm (ϵ 8300 M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 2.92 (m, 8, CH_2CH_2), 3.46 (m, 4, CH_2CH_2), 4.68 (s, 2, $ArCH_2$), 7.52 (m, 4, ArH), 8.01 (d, 2, ArH), 8.34 (d, 2, ArH), 8.49 (s, 1, ArH); ^{13}C NMR (D_2O) δ 43.37, 44.43, 46.06, 53.22, 124.86, 126.29, 127.83, 128.24, 129.46, 130.27, 131.49, 131.91; FAB mass spectrum, *m/e* 319 (M^+); high-resolution FAB mass spectrum of free base calcd for $C_{21}H_{25}N_3$ 319.205, measd 319.205; molecular formula based on best fit to the microanalytical data, $C_{21}H_{25}N_3 \cdot 3HCl \cdot 2H_2O$.

9-(1′,4′,7′,10′-Tetraazacyclododecyl)methylanthracene (3c). 1,4,7,10-Tetraazacyclododecane (**2c**; 0.86 g, 5 mmol) and 9-(chloromethyl)anthracene (0.23 g, 1 mmol) were reacted as described for **3a** to afford anthrylazamacrocycle **3c** (0.27 g, 75%): mp 182 °C dec; UV (λ_{max}[pH 7]) 370 nm (ϵ 8000 M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 2.85 (br m, 16, CH_2CH_2), 4.70 (s, 2, $ArCH_2$), 7.55 (m, 4, ArH), 8.09 (m, 4, ArH), 8.53 (s, 1, ArH); ^{13}C NMR (D_2O) δ 42.59, 43.03, 44.75, 50.54, 50.88, 123.79, 126.46, 127.45, 128.41, 130.10, 130.75, 131.21, 132.09; FAB mass spectrum, *m/e* 362 (M^+); high-resolution FAB mass spectrum of free base calcd for $C_{23}H_{30}N_4$ 362.247, measd 362.250; molecular formula based on best fit to the microanalytical data, $C_{23}H_{30}N_4 \cdot 3.1HCl \cdot 2H_2O$.

9-(1′,4′,7′,10′,13′-Pentaazacyclopentadecyl)methylanthracene (3d). 1,4,7,10,13-Pentaazacyclopentadecane (**2d**), obtained by the described extraction of 1,4,7,10,13-pentaazacyclopentadecane pentahydrochloride (1.1 g, 2.7 mmol), was dissolved in warm toluene (12 mL) and the resulting solution was placed in a pressure tube. 9-(Chloromethyl)-anthracene (0.152 g, 0.67 mmol) was added to the solution and the reaction mixture was heated in a 65 °C oil bath with stirring for 1 day. After being cooled, the reaction mixture was extracted with 5% aqueous sodium hydroxide (2 × 5 mL) and then with water (2 × 5 mL). The organic layer was dried over sodium carbonate then evaporated to dryness in vacuo. The resulting residue was dissolved in ethanol (20 mL) and a few drops of concentrated hydrochloric acid were added to yield the crude hydrochloride salt (0.21 g), which was collected by filtration and dried in vacuo. ^1H NMR showed this sample to contain 0.2 equiv of the pentaazamacrocycle **2d**. An additional extraction of the crude salt between 10% aqueous sodium carbonate and chloroform followed by reprecipitation of the hydrochloride salt from ethanol yielded **3d** as a yellow solid (0.153 g, 40%), which was shown by ^1H NMR to contain 0.03 equiv of **2d**: mp 160 °C dec; UV (λ_{max}[pH 7]) 370 nm (ϵ 7700 M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 2.9 (m, 20, CH_2CH_2), 3.14 (s, 0.6, free pentacyclen), 4.82 (s, 2, $ArCH_2$), 7.6 (m, 4, ArH), 8.13 (d, 2, ArH), 8.38 (d, 2, ArH), 8.64 (s, 1, ArH); ^{13}C NMR (D_2O) δ 44.62, 45.53, 45.82 (free pentacyclen overlaps with this peak), 46.14, 52.13, 52.35, 124.74, 126.50, 128.19, 130.10, 130.57, 131.74, 132.16;[21] FAB mass spectrum, *m/e* 406 ($M + 1)^+$; high-resolution FAB mass spectrum of free base calcd for $C_{25}H_{36}N_5$ 406.297, measd 406.300; molecular formula based on best fit to the microanalytical data, $C_{25}H_{35}N_5 \cdot 3.5HCl \cdot 2H_2O + 0.03C_{10}H_{25}N_5$.

9-(1′,4′,7′,10′,13′,16′-Hexaazacyclooctadecyl)methylanthracene (3e). 1,4,7,10,13,16-Hexaazacyclooctadecane (**2e**), obtained by the described extraction of 1,4,7,10,13,16-hexaazacyclooctadecane trisulfate (0.91 g, 1.6 mmol), was dissolved in warm acetonitrile (60 mL) and 9-(chloromethyl)anthracene (0.073 g, 0.32 mmol) was added. The reaction mixture was stirred in a pressure tube with heating (60 °C oil bath) overnight while protected from light. After cooling, the solvent was removed in vacuo. The residue was partitioned between 10% aqueous sodium carbonate (20 mL) and toluene (75 mL), and the organic layer was dried over potassium carbonate and evaporated to dryness *in vacuo*. The residue was suspended in ethanol and vacuum filtered, and then concentrated hydrochloric acid was added dropwise to the filtrate to yield the crude hydrochloride salt (0.144 g) after filtering and drying. An additional extraction of the regenerated free base using chloroform and 10% aqueous sodium carbonate followed by reprecipitation of the hydrochloride salt from ethanol yielded **3e** as a yellow solid (0.089 g, 40%), which was shown by ^1H NMR to contain 0.04 equiv of **2e**: mp 190 °C dec; UV (λ_{max}[pH 7]) 370 nm (ϵ 7300 M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 3.0 (br m, 23 (24 expected), CH_2CH_2), 3.14 (s, 0.96, free hexacyclen), 4.6 (br s, HOD; obscures $ArCH_2N$), 7.55 (m, 4, ArH), 8.08 (d, 2, ArH), 8.41 (d, 2, ArH), 8.56 (s, 1, ArH); ^{13}C NMR (D_2O) δ 44.91, 46.22, 46.36 (free hexacyclen overlaps with this peak), 46.73, 47.39, 51.79, 52.18, 125.33, 126.54, 128.18, 129.78, 130.37, 131.78, 132.14;[21] FAB mass spectrum of the free base, *m/e* 449 ($M + 1)^+$; high-resolution FAB mass spectrum calcd for $C_{27}H_{41}N_6$ 449.339, measd 449.338; molecular formula based on best fit to the microanalytical data, $C_{27}H_{40}N_6 \cdot 5HCl \cdot 3H_2O + 0.04C_{12}H_{30}N_6$.

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(21) This CMR spectrum revealed one less aromatic carbon than expected, likely buried under one of the observed aromatic aks.