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Complexation of Creatinine by Synthetic Receptors

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Abstract: A series of highly preorganized, polyheterocyclic hosts for creatinine has been synthesized. Key receptors bearing intrinsic chromophores are ketones **4** and **9**. ^{1}H NMR and UV-visible spectroscopy were used to detect extraction of creatinine hydrochloride by **4** into $CDCl_3$ and 5% ethanol/chloroform, respectively. Receptor **9**, which has an additional hydrogen-bond donor, binds creatinine in 70 % aq. methanol, as shown by changes in UV-visible absorptions at constant pH* (4.3). These hexagonal lattice receptors are the most effective complexing agents known to bind creatinine, which is a clinically important constituent of blood.

Chromophore-containing complexing agents are attractive targets for designed molecular recognition because they have potential for immediate practical application. Chromogenic (color-producing) receptors promise to replace analytical reagents that generate color changes by irreversible chemical reactions.[1] Particularly good progress has been made in the development of chromogenic ionophores[2,3] (chromoionophores[3f]) that change color upon reversible complexation of metal ions. Conceptually related approaches to measurement of organic analytes employ dye chromophores or fluorophores conjugated to biomolecular receptors, such as antibodies.[4] In such conjugates, the reporter subunit often is loosely linked (extrinsic) to the binding site, resulting in weak optical response. Reported here is a series of synthetic receptors in which the reporter chromophore is *intrinsic* to the molecular site that binds an organic analyte.

Urea and creatinine are blood metabolites of considerable importance in clinical chemistry, particularly as indicators of renal function.[5] Routine clinical analysis of creatinine in blood is currently conducted colorimetrically by means of the Jaffé reaction or by enzymatic methods. While the Jaffé reaction is not specific for creatinine, enzymatic methods are more expensive and are subject to interference by certain pharmaceuticals.[5] The purpose of this study was to determine whether the hexagonal lattice approach to hydrogen-bonded complexation of organic molecules[6] could be applied to creatinine. These fused-ring heterocycles have chromophores, often also fluorophores, that are intrinsic to the binding site.[6a] Recently an alternative approach was reported for hydrogen-bonded complexation of creatinine by simpler, less highly preorganized synthetic receptors.[7]

A previously reported hexagonal lattice diketone extracts urea from water into chlorinated solvents.[6e] In structure **1** proposed for this complex (Figure 1), the two carbonyl oxygen atoms and two of the four nitrogen atoms act as hydrogen-bond acceptors (A), forming the primary contacts with the urea NH_2 groups.

The remaining two nitrogen atoms of the "spacer" pyridine rings of the receptor may further stabilize the complex by secondary electrostatic interactions[8] indicated by additional dotted lines in diagram **A** (Figure 1).

Creatinine has three different tautomeric/stereoisomeric forms of similar energy[9] and the hydrogen-bond donor-acceptor arrays required to bind each of these forms are shown in diagrams **B-D** (Figure 1). Array **B** was chosen for receptor design because it has the fewest destabilizing secondary electrostatic interactions[8] and we anticipated that one donor group (D-H) could be introduced by protonation of a pyridine nitrogen atom under mildly acidic conditions. The specific receptor (**9-H$^+$**) and the proposed structure of the creatinine complex are shown in Figure 1.

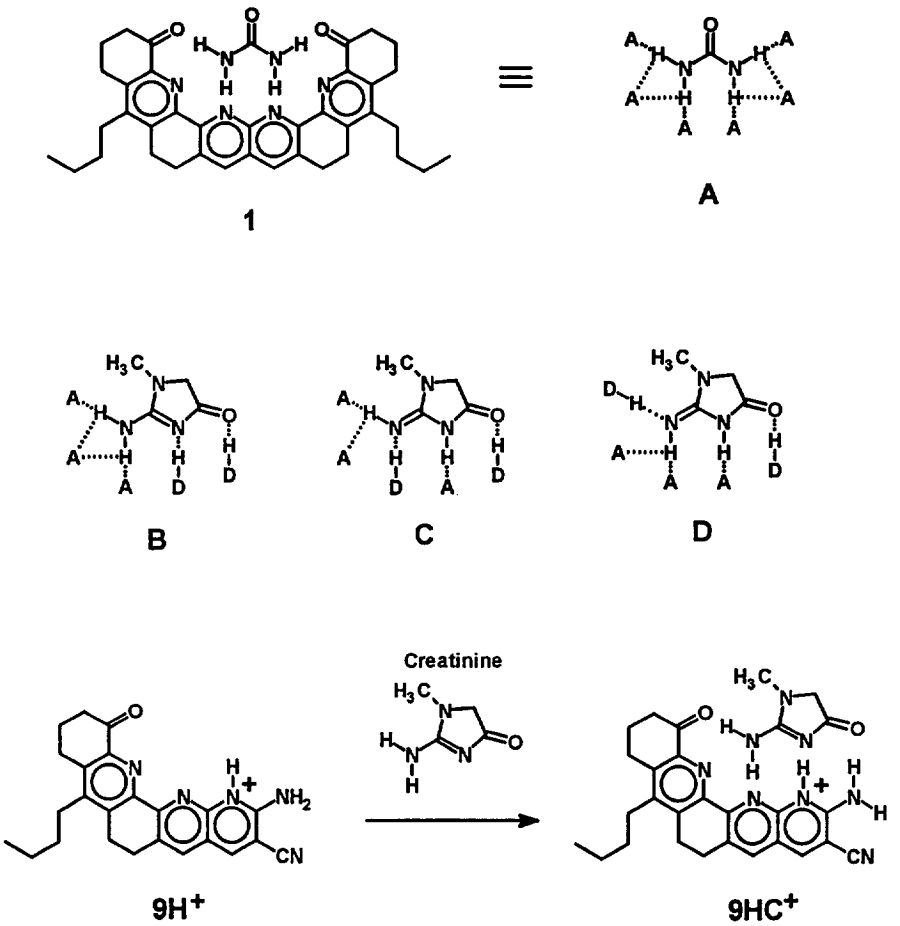

Figure 1. Conceptual approach to complexation of creatinine by protonated receptor **9H$^+$**.
A: arrangement of hydrogen bond acceptors (A) in urea complex **1**.[6e] **B-D**: alternatives for complexation of creatinine by receptors containing hydrogen-bond donors (DH) and acceptors. Attractive secondary electrostatic interactions are illustrated by long dotted lines.

SYNTHESIS

The routes used to synthesize the designed creatinine receptor (**9**) and related compounds are shown in Figure 2. The key intermediate in these syntheses is 5-benzylidene-9-butyl-2,3,5,6,7,8-hexahydroacridin-4(1H)-one (**2**),which is available in five steps from inexpensive starting materials.[6d,10] Friedländer

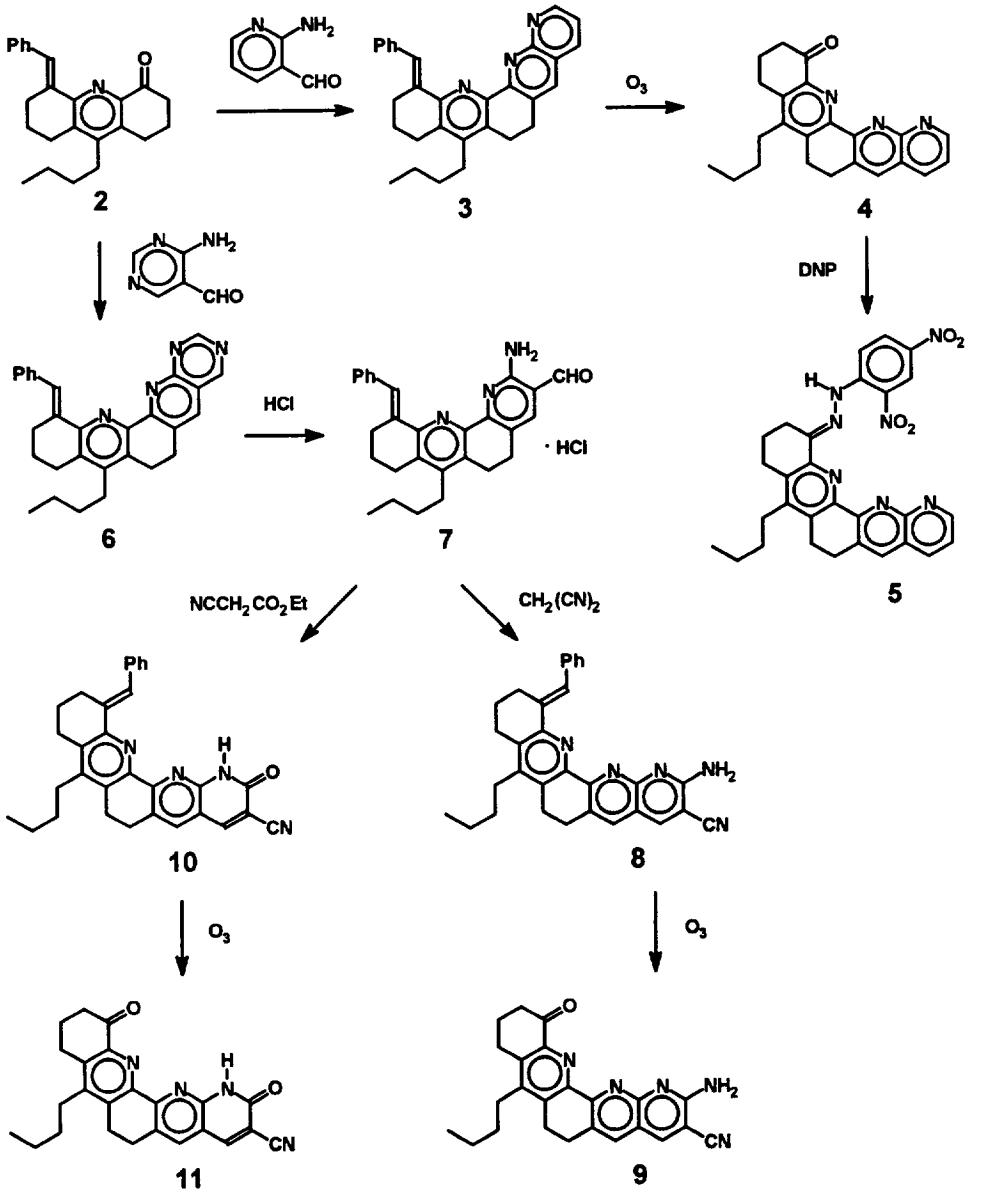

Figure 2. Syntheses of creatinine receptors **4** and **9** and related compounds. See Experimental section for details.

condensation[11] of this ketone with 2-aminonicotinaldehyde[12] or 5-aminopyrimidine-4-carboxaldehyde[13] gave the 1,8-naphthyridine **3** or the 8-azaquinazoline **6**,[6c] respectively, in high yields. Ozonolysis of **3** in CH_3OH/CH_2Cl_2, followed by dimethylsulfide workup, gave ketone **4**, which was of interest as a creatinine receptor missing the NH_2 hydrogen-bond donor present in **9**. This ketone was also characterized as DNP derivative **5**, which has an extended chromophore. As previously described in the synthesis of the diketone receptor in urea complex **1**[6e], the pyrimidine ring of **6** can be hydrolyzed in aqueous HCl to give aminoaldehyde **7**. Alkaline condensation of **7** with malononitrile or ethyl cyanoacetate by the method of Gorecki and Hawes[14] gave aminonitrile **8** or 3-cyano-2-pyrimidone **10**, respectively. Ozonolysis of **8** or **10**, as described above for **3**, afforded candidate creatinine receptors **9** or **11**, respectively.

COMPLEXATION

Initial creatinine complexation studies focused on ketone **4** and DNP derivative **5**. Protonation of the terminal pyridine ring of either compound, as in $\mathbf{9H^+}$, would permit complexation of neutral creatinine, as in $\mathbf{9H^+}$•creatinine (Figure 1). Alternately, the neutral receptors could bind protonated creatinine. Qualitative proof that **4** binds protonated creatinine was obtained by means of ion-pair extraction using bromocresol green (BCG) as the counterion. In this experiment an aqueous solution of creatinine (0.01 M) and BCG (6 x 10^{-5} M) in 4-morpholinethanesulfonate (MES) buffer (0.1 M, pH 5.5) was extracted with a solution of receptor **4** (10^{-3} M) in CH_2Cl_2. Depletion of BCG^- from the aqueous solution caused a decrease of the absorbance at 616 nm from 1.43 to 0.78.

Binding of creatinine•H^+ by ketone **4** was also shown by solid-liquid extraction experiments employing ^{1}H-NMR and UV-visible spectroscopy. Addition of an excess of solid creatinine hydrochloride to a solution of **4** in $CDCl_3$ (6 x 10^{-3} M) resulted in extraction of creatinine•HCl, as shown by the ^{1}H-NMR spectra in Figure 3. The integrals of the creatinine methylene and methyl signals at 3.5 and 4.4 ppm, respectively, indicated that 0.4-0.5 equivalents of the guest are extracted in this experiment, whereas creatinine signals could not be detected in control experiments omitting **4**. Complexation is also shown by slight downfield shifts of the receptor ^{1}H-NMR signals, particularly for the terminal pyridine ring. Addition of excess solid creatinine to a 5 x 10^{-5} M solution of **4** in 5% ethanol/chloroform caused a 4 nm bathochromic shift of the absorption maximum at 350 nm (Figure 4), showing that **4** binds creatinine to some extent even in a hydrogen-bonding solvent. Experiments with DNP derivative **5** were limited by its poor solubility in organic solvents, but extraction of solid creatinine hydrochloride into a 5.5 x 10^{-5} M solution of **5** in 5% ethanol/chloroform caused a slight increase of the 352 nm absorption and a decrease of intensity at the secondary λ_{max} at 392 nm.

Receptors **9** and **11** are expected to bind protonated creatinine more strongly than **4** because the complexes should have an additional hydrogen bond. Protonation of these receptors to form $\mathbf{9•H^+}$ and $\mathbf{11•H^+}$ offers the possibility of binding neutral creatinine, shown in hydrogen-bonding array **B** (Figure 1). Receptor **9** appeared a better candidate for complexation of neutral creatinine because protonation on nitrogen rather than oxygen should give a more stable complex. For this reason and because solutions of **11** were not stable over long periods of time, receptor **9** was chosen for creatinine binding studies.

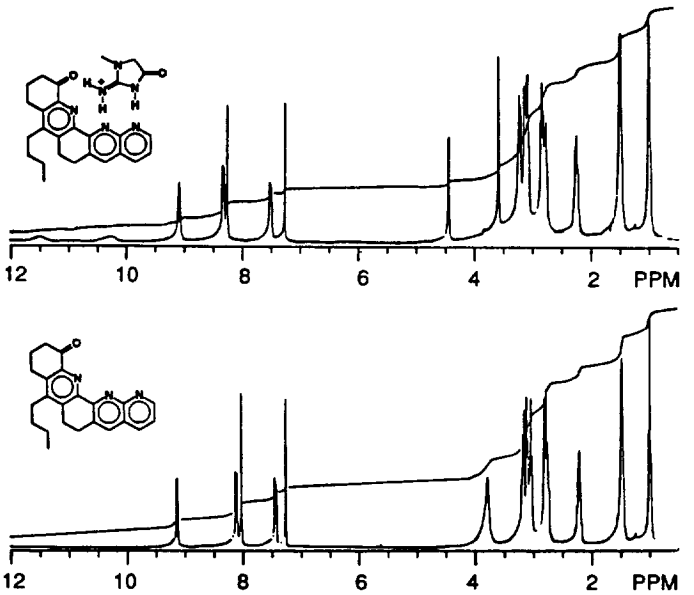

Figure 3. 300 MHz ^{1}H NMR spectra of solutions of receptor **4** in $CDCl_3$ (6 x 10^{-3} M) before and after exposure to solid creatinine hydrochloride.

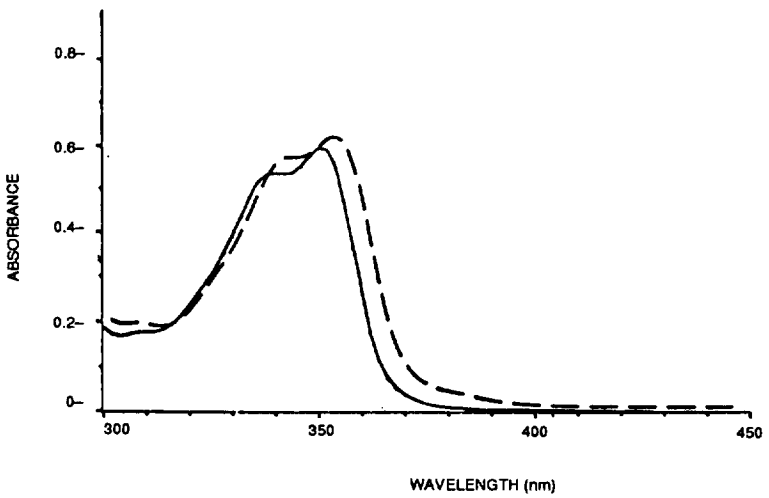

Figure 4. UV-visible spectra of solutions of receptor **4** in 95:5 (v/v) chloroform/ethanol before (solid line) and after (dashed line) exposure to solid creatinine hydrochloride.

Preliminary experiments showed that addition of creatinine to methanolic solutions of **9**•**HCl** caused quenching of the yellow-green fluorescence (λ_{em}~490 nm, λ_{ex} 408 nm). The absorption maximum at 442 nm was also decreased in intensity. These effects were qualitatively reproduced by addition of bases to methanolic **9**•**H**$^+$, so careful consideration of acid-base equilibria was required. Important competing acid-base and complexation equilibria are shown in equations 1-3, where C = creatinine, R = **9**, K_R and K_c are the acidity constants of **9**•**H**$^+$ and creatinine hydrochloride, respectively, and K_s is the stability constant of **RH**$^+$•creatinine. Note that **RH**$^+$•creatinine can be formed from **RH**$^+$ and creatinine or from free base receptor (**R**) and creatinine•H$^+$ and that the stability constant can be defined either way.

$$CH^+ \overset{K_c}{\rightleftharpoons} C + H^+ \qquad (1)$$

$$RH^+ \overset{K_{RH}}{\rightleftharpoons} R + H^+ \qquad (2)$$

$$RH^+ + C \overset{K_s}{\rightleftharpoons} RHC^+ \qquad (3)$$

The pK_a values for creatinine•HCl (pK_c, Eq. 1) in methanol and 70 % aq. methanol were estimated by means of the indicator neutral red.[15] Both pK_c values (methanol, 3.4; 70% aq. methanol, 4.2) were found to be lower than the pK_a of creatinine in water (4.5-5.0),[16] whereas a pK_c value of 4.9 was measured by potentiometric titration of creatinine in 70% aq. methanol.[17] Measurement of pK_R defined in Eq. 2 proved to be much more difficult because **9** can undergo multiple protonation. As described in the experimental section, treatment of **9** with 2 N aq. HCl initially yields solid **2**$(HCl)_2$, which loses HCl upon heating under vacuum. Treatment of **9** with dilute methanolic HCl gives **9**•HCl containing 1.25 equivalents of H_2O. Drying **9**•HCl to consistent water content presents further difficulty, since heating at 80 °C under vacuum for 14 hours causes nearly complete loss of HCl. The low solubility of **9** in methanol and aq. methanol precluded determination of ionization constants by potentiometric titration, and spectrophotometric methods[18] could not be used because the molar absorptivities of **9**•**H**$^+$ and **9**$(H^+)_2$ could not be determined accurately.

The stability constant (K_s) of **9HC**$^+$ cannot be calculated without knowledge of K_{RH}, but qualitative proof of complexation could be obtained by monitoring absorbance changes upon addition of creatinine to pH-controlled solutions of **9**. At constant pH the ratio **9**/**9H**$^+$ remains constant and changes in absorbance can be attributed to the formation of **9HC**$^+$. Initial experiments were conducted with MES-buffered aqueous methanol solutions of **9**, but addition of creatinine caused the greatest absorbance changes near pH 4, which lies outside the buffering range of MES. Hence, a constant pH* of 4.3 was obtained by addition of HCl after addition of various quantities of creatinine to solutions of **9** in 70% aq. methanol. Figure 5 shows that incremental addition of creatinine to **9** at pH* 4.3 caused progressive decreases in the intensities of the 422 and 442 nm peaks; the absorbance effects are shown graphically in Figure 6.

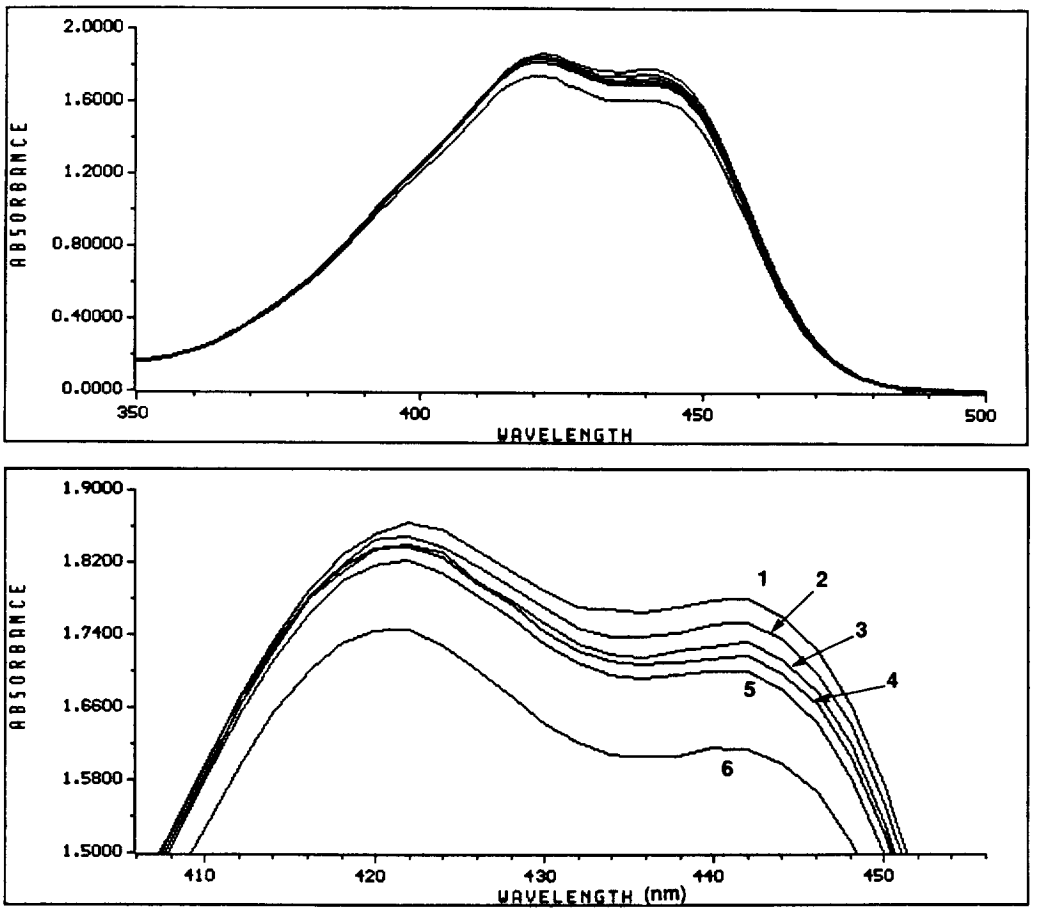

Figure 5. UV-visible spectra of pH* 4.3 solutions of receptor **9** (8.1×10^{-5} M) in 70:30 (w/w) methanol/water containing the following concentrations of creatinine: 1, 0 mM; 2, 3.6 mM; 3, 5.4 mM; 4, 7.2 mM; 5, 9.0 mM; 6, 14.4 mM.

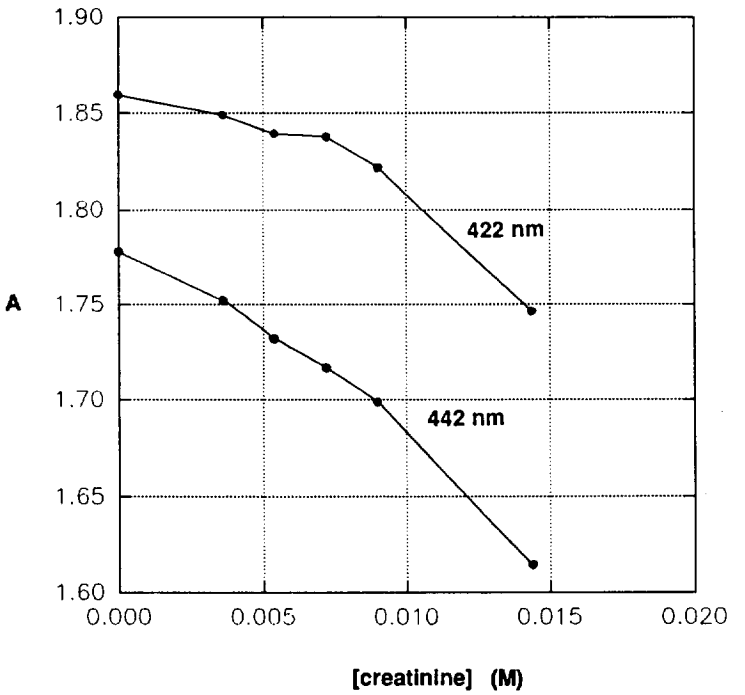

Figure 6. Graph of absorbances at λ_{max} values for spectra shown in Figure 5 vs. creatinine concentrations.

CONCLUSION

The key results of these studies are the qualitative observation of creatinine binding by receptor **4** in 5% ethanol/chloroform and by receptor **9** in 70% aq. methanol. These results show that hexagonal lattice receptors constitute a more effective approach to complexation of creatinine than less highly preorganized systems.[7] The latter hosts have only been shown to bind a lipophilic analogue of creatinine in nonpolar aprotic solvents. Moreover, chromophores present in **4** and **9** signal the complexation of creatinine. Efforts are in progress to enhance this chromogenic response, leading to potential applications of hexagonal lattice receptors as creatinine reagents or sensors.[19]

EXPERIMENTAL

12-Benzylidene-8-n-butyl-6,7,9,10,11,12-hexahydrobenzo[b]pyrido[3,2-j][1,10]-phenanthroline (3). To a 250 mL round-bottomed flask equipped with a side arm, septum, stir bar, condenser and N_2 inlet were added 3.10 g (9.0 mmol) of 5-benzylidene-9-n-butyl-2,3,5,6,7,8-hexahydro-4(1H)-acridinone (**2**),[10] 1.12 g (9.2 mmol) of 2-aminonicotinaldehyde[12] and 75 mL of MeOH. The apparatus was flushed with N_2 then 0.2 mL of 15 % (w/w) KOH/MeOH was added by syringe and the reaction mixture was heated under reflux. Heating was continued under N_2 for 24 h and the reaction mixture was allowed to cool to room temperature. Vacuum filtration and washing with cold MeOH (20 mL) followed by drying at room temperature (0.5 mm, 7 h) yielded 3.30 g (90 %) of the product as a yellow solid that was suitable for use in the next step without purification. Analytically pure material was obtained by recrystallization from EtOH followed by drying at 78 °C (0.3 mm, 40 h), mp 130-132 °C. ^{1}H NMR ($CDCl_3$, 7.26 ppm) δ 9.10 (dd, J = 2, 4 Hz, H2, 1 H), 8.44 (s, ArCH, 1 H), 8.12 (dd, J = 2, 8 Hz, H4, 1 H), 7.99 (s, H5, 1 H), 7.20-7.49 (m, ArH, 5 H), 7.42 (dd, J = 4, 8 Hz, H3, 1 H), 3.13-3.18 (m, H6, 2 H), 3.02-3.07 (m, H7, 2 H), 2.86-2.92 (m, H9, CH_2-propyl, 4 H), 2.71 (t, J = 6 Hz, H11, 2 H), 1.88 (m, H10, 2 H), 1.47 (m, $CH_2CH_2CH_3$, 4 H), 0.99 (t, J = 7 Hz, CH_3, 3 H); ^{13}C NMR ($CDCl_3$, 77.0 ppm) δ 156.0, 155.5, 152.3, 151.6, 147.7, 147.0, 138.2, 135.7, 135.3, 134.0, 132.7, 132.0, 131.6, 129.5, 127.5, 126.0, 122.2, 121.3, 30.7, 27.8, 27.7, 27.3, 26.4, 23.4, 22.7, 22.5, 13.5; IR (KBr) 3058 (w), 2930 (s), 2860 (m) 1645 (m), 1604 (m), 1540 (s), 1447 (s), 1377 (s), 1214 (m), 1144 (m), 1034 (m), 900 (m), 789 (m), 691 (m), cm^{-1}; EI-MS m/z 431 (M^+, 5), 388 (4), 249 (4 %). Anal. Calcd for $C_{30}H_{29}N_3 \cdot 1H_2O$: C, 80.15, H, 6.95, N, 9.35; Found: C, 79.84, H, 7.04, N, 9.14 %.

8-n-Butyl-6,7,10,11-tetrahydrobenzo[b]pyrido[3,2-j][1,10]phenanthrolin-12(9H)-one (4). To a 250 mL round-bottomed flask were added 0.31 g (0.72 mmol) of 12-benzylidene-8-n-butyl-6,7,9,10,11,12-hexahydrobenzo[b]pyrido[3,2-j][1,10]phenanthroline (**3**), 30 mL of CH_2Cl_2 and 60 mL of CH_3OH. The resulting solution was cooled to ca. -78 °C by means of a dry ice/acetone bath and N_2 was bubbled through the solution for 5 m. A stream of O_3/O_2 was then bubbled through the solution until it became dark blue-green. The solution was purged by bubbling N_2 through it for an additional 5 m, then 0.3 mL of dimethyl sulfide was added by syringe. The reaction mixture was allowed to warm to room temperature overnight and the solvents were removed by rotary evaporation. The oily residue was washed with ether (3 x 50 mL) and recrystallized by slow evaporation of a solution in CH_2Cl_2/isopropanol (50 mL, ~5:1). Vacuum filtration yielded 0.14 g (53 %) of product as a yellow solid after drying at 78 °C (0.3 mm, 24 h), mp 207-209 °C (dec). ^{1}H NMR ($CDCl_3$), δ 9.13 (dd, J = 2, 4 Hz, H2, 1 H), 8.11 (dd, J = 2, 8 Hz, H4, 1 H), 8.02 (s, H5, 1 H), 7.44 (dd, J = 4, 8 Hz, H3, 1 H), 3.16-3.19 (m, H6, 2 H), 3.11 (m, H7, 2 H), 3.04 (t, J = 6 Hz, H11, 2 H), 2.74-2.83 (m, H9, CH_2-propyl, 4 H), 2.21 (m, H10, 2 H), 1.47 (m, $CH_2CH_2CH_3$, 4 H), 0.99 (t, J = 7 Hz, CH_3, 3 H); ^{13}C NMR ($CDCl_3$) δ 196.9, 155.6, 154.8, 153.5, 149.6, 148.8, 147.2, 139.7, 137.4, 135.9, 135.0, 132.3, 122.8, 122.2, 39.2, 31.0, 28.3, 27.4, 26.1, 24.3, 23.0, 22.1, 13.8; IR (KBr) 2955 (m), 2908 (m), 2872 (w), 1690 (s), 1613 (m), 1561 (m), 1455 (m), 1167 (m), 1102 (m), 914 (w), 797 (m) cm^{-1}; UV-visible ($CHCl_3$) λ_{max} (log ε) 244 (4.5), 340 (4.2), 350 nm (4.3); EI-MS m/z 357 (M^+, 100), 328 (43), 314 (15 %). Anal. Calcd for $C_{23}H_{23}N_3O \cdot 1.25H_2O$: C, 72.70; H, 6.76; N, 11.06; Found: C, 72.92; H, 6.56; N, 10.89 %.

8-n-Butyl-6,7,10,11-tetrahydrobenzo[b]pyrido[3,2-j][1,10]phenanthrolin-12(9H)-one dinitrophenylhydrazone (5). To a 250 mL round-bottomed flask fitted with a stir bar and N_2 inlet were added 0.132 g (0.37 mmol) of 8-n-butyl-6,7,10,11-tetrahydrobenzo[b]pyrido[3,2-j][1,10]phenanthrolin-12(9H)-one (**4**), 10 mL of CH_2Cl_2 and 30 mL of EtOH. Dinitrophenylhydrazine (0.077 g, 0.39 mmol) was added and the apparatus was flushed with N_2. The resulting solution was stirred at room temperature for 36 h then concentrated by rotary evaporation. A suspension of the crude product in boiling EtOH was cooled to room temperature and vacuum filtered, yielding 0.15 g (75 %) of product as an orange solid after drying at 78 °C (0.3 mm, 24 h), mp > 220 °C. ^{1}H NMR ($CDCl_3$), δ 11.49 (bs, NH, 1 H), 9.17 (m, H2, 1 H), 9.14 (m, DNPH3, 1 H), 8.62 (m, DNPH5, 1 H), 8.45 (m, DNPH6, 1 H), 8.16 (m, H4, 1 H), 8.05 (s, H5, 1 H), 7.48 (m, H3, 1 H), 3.17-3.20 (m, H6, 2 H), 3.11-3.16 (m, H7, 2 H), 2.91-2.95 (m, H9,11, 4 H), 2.70 (m, CH_2-propyl, 2 H), 2.22 (m, H10, 2 H), 1.49 (m, $CH_2CH_2CH_3$, 4 H), 0.99 (t, J = 7 Hz, CH_3, 3 H); UV-visible ($CHCl_3$) λ_{max} (log ε) 352 (4.2), 390 nm (4.5); FAB-MS m/z 538 (M+1, 100 %). Anal. Calcd for $C_{29}H_{27}N_7O_4 \cdot 1H_2O$: C, 62.69; H, 5.26; N, 17.65; Found: C, 62.31; H, 5.23; N, 17.53 %.

2-Amino-12-benzylidene-8-n-butyl-3-cyano-6,7,9,10,11,12-hexahydrobenzo[b]pyrido[2,3-j][1,10]phenanthroline (8). To a 100 mL round-bottomed flask equipped with a stir bar, condenser and N_2 inlet were added 3.77 g (8.20 mmol) of 2-amino-11-benzylidene-7-n-butyl-5,6,8,9,10,11-hexahydrobenzo[b][1,10]phenanthroline-3-carboxaldehyde•HCl (**7**),[6c] 50 mL of anhydrous ethanol, 1.84 g (27.9 mmol) of malononitrile and 1.0 mL (10 mmol) of piperidine. The apparatus was flushed with N_2 then the reaction mixture was heated under reflux for 6 h. The mixture was cooled to room temperature and then to 0 °C. A yellow solid was collected by filtration, washed with cold ethanol (2 x 5 mL) and dried to give 3.52 g (91 %) of product that was pure by ^{1}H NMR, mp 196 °C (dec). A sample for microanalysis was purified by recrystallization from a mixture of ethanol and 1-propanol and dried at 61 °C (0.5 mm, 24 h). ^{1}H NMR ($CDCl_3$), δ 8.35 (s, H5, 1 H), 8.23 (s, ArCH, 1 H), 7.81 (s, H4, 1H), 7.22-7.47 (m, ArH, 5 H), 5.65 (s, NH_2, 2 H), 2.85-3.05 (m, H6,7,9,11 8 H), 2.71 (t, CH_2-propyl, 2 H), 1.90 (m, H10, 2 H), 1.48 (m, $(CH_2)_2CH_3$; 4 H), 1.00 (s, CH_3, 3 H); ^{13}C NMR ($CDCl_3$), δ 159.0, 157.4, 157.0, 152.5, 148.0, 147.3, 143.5, 138.5, 135.7, 134.4, 132.6, 132.5, 130.2, 129.8, 128.4, 127.8, 126.4, 116.0, 115.8, 95.6, 31.1, 28.2, 27.8, 27.6, 26.8, 24.0, 23.2, 22.9, 13.9; IR (KBr) 3340 (m), 3194 (w), 2952 (m), 2929 (m), 2864 (w), 2218 (m), 1612 (s), 1536 (m), 1481 (m), 1439 (m), 1215 (w), 805 (w), 695 (m) cm^{-1}. FAB-MS m/z 472 (M+1, 100 %); Anal. Calcd for $C_{31}H_{29}N_5 \cdot 0.75\ H_2O$: C, 76.75; H, 6.34; N, 14.44; Found: C, 76.59; H, 6.20; N, 14.48 %.

2-Amino-8-n-butyl-3-cyano-6,7,10,11-tetrahydrobenzo[b]pyrido[2,3-j][1,10]phenanthrolin-12(9H)-one (9). To a 1 L round-bottomed flask were added 1.00 g (2.12 mmol) of crude 2-amino-12-benzylidene-8-n-butyl-3-cyano-6,7,9,10,11,12-hexahydrobenzo[b]pyrido[2,3-j][1,10]phenanthroline (**8**), 500 mL of CH_2Cl_2 and 300 mL of CH_3OH. The resulting solution was cooled to approximately -78 °C by means of a dry-ice/acetone bath and N_2 was bubbled through it for 5 m. A stream of O_3/O_2 was bubbled through the solution until the color changed from orange-yellow to greenish-yellow. N_2 was then bubbled through the solution for an additional 5 m, then 2.0 mL of dimethyl sulfide was added via syringe. The solution was allowed to warm to room temperature overnight and the solvents were removed by rotary evaporation. The crude product was washed with ether (3 x 100 mL) then recrystallized from n-BuOH/CH_3CN. Vacuum filtration gave 0.75 g

(89 %) of product as a yellow crystalline solid after drying at 61 °C (0.5 mm, 48 h), mp > 300 °C. ^{1}H NMR (DMSO-d_6, 2.49 ppm) δ 8.70 (s, H4, 1 H), 8.03 (s, H5 1 H), 7.21 (bs, NH_2, 2 H), 3.02-3.06 (m, H6,7,11, 6 H), 2.73 (m, CH_2-propyl, H9, 4 H), 2.12 (m, H10, 2 H), 1.44 (m, $CH_2CH_2CH_3$, 4 H), 0.94 (t, J = 7 Hz, CH_3, 3 H); ^{18}C NMR ($CDCl_3$), 196.1, 157.8, 156.8, 156.4, 149.2, 148.3, 146.9, 145.5, 140.1, 137.8, 135.2, 129.0, 116.2, 115.2, 95.3, 30.3, 27.6, 26.2, 25.5, 23.8, 22.4, 21.7, 13.6; IR (KBr) 3406 (w), 2953 (w), 2215 (w), 1654 (m), 1636 (s), 1628 (s), 1624 (s), 1616 (s), 1610 (s), 1559 (s), 1437 (m), 1221 (m), 1180 (m), 803 (w) cm^{-1}; FAB-MS m/z 398 (M + 1, 100 %). Anal. Calcd for $C_{24}H_{23}N_5O$•0.25 H_2O: C, 71.71; H, 5.89; N, 17.42; Found: C, 71.51; H, 5.81; N, 17.41 %.

2-Amino-8-n-butyl-3-cyano-6,7,10,11-tetrahydrobenzo[b]pyrido[2,3-j][1,10]phenanthrolin-12(9H)-one hydrochloride (9•HCl). Method A. A suspension of 0.14 g (0.35 mmol) of analytically pure **9** in 20 mL of 2 N aq. HCl was stirred for 2 h at rt under N_2. The resulting solution was concentrated to dryness by rotary evaporation. The residue was dried for 16 h at rt (1 mm) to afford 0.16 g (95 %) of **2**•$(HCl)_2$ as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 9.08 (s, 1 H, H4), 8.77 (bs, 2 H, NH_2), 8.53 (s, 1 H, H5), 3.04-3.25 (m, 6 H, H6,7, CH_2-propyl), 2.22-2.85 (m, 4 H, H9,11), 2.14 (m, 2 H, H10), 1.46 (m, 4 H, $CH_2CH_2CH_3$), 0.95 (t, 3 H, CH_3). Anal. calcd for $C_{24}H_{25}N_5OCl_2$•1.5H_2O: C, 57.96; H, 5.67; N, 14.08; Cl, 14.25. Found: C, 57.92; H, 5.53; N, 14.09; Cl, 14.73 %. Drying at 80 °C (1 mm) gave a pure sample of **9**•HCl. Anal. Calcd for $C_{24}H_{24}N_5OCl$: C, 66.43; H, 5.57; N, 16.14. Found: C, 66.74; H, 5.66; N, 16.07 %. **Method B.** A solution of 0.10 g (0.25 mmol) of pure **9** and 30 mg (0.36 mmol) of con. aq. HCl in 12 mL of methanol was stirred for 3 h at rt under N_2. The reaction mixture was filtered and the filtrate was added slowly to 100 mL of rapidly stirred ether. The resulting precipitate was isolated by centrifugation, washed with ether (2 x 50 mL) and dried for 16 h at rt (1 mm), yielding 96 mg (86 %) of product as a yellow solid. Anal. calcd for $C_{24}H_{24}N_5OCl$ •1.25H_2O: C, 63.15; H, 5.85; N, 15.34; Cl, 7.77; Found: C, 63.33; H, 5.85; N, 15.24; Cl, 7.40 %.

12-Benzylidene-8-n-butyl-3-cyano-6,7,9,10,11,12-hexahydrobenzo[b]pyrido[2,3-j][1,10]phenanthrolin-2(1H)-one (10). To a 100 mL round-bottomed flask equipped with a stir bar, condenser and N_2 inlet were added 2.01 g (4.37 mmol) of 2-amino-11-benzylidene-7-n-butyl-5,6,8,9,10,11-hexahydrobenzo[b] [1,10]phenanthroline-3-carboxaldehyde•HCl (**7**),[6e] 75 mL of anhydrous ethanol and 1.8 mL (16.9 mmol) of ethyl cyanoacetate. The apparatus was flushed with N_2, the reaction mixture was heated under reflux and 1.0 mL (10 mmol) of piperidine was added by syringe. Heating was continued under nitrogen for 6 h, then the mixture was cooled to room temperature and finally to 0 °C for 1 h. Vacuum filtration and washing with cold absolute ethanol (2 x 5 mL) yielded 1.81 g (88 %) of yellow product after drying at room temperature (0.8 mm, 24 h). Recrystallization from CH_3Cl/EtOH gave analytically pure **10**, mp 291 °C (dec.). ^{1}H NMR ($CDCl_3$), δ 7.77 (s, ArCH, 1 H), 7.73 (s, H5, 1 H), 7.28 (s, H4, 1 H), 7.04-7.23 (m, ArH, 5 H), 2.95-3.03 (m, H6,7, 4 H), 2.85-2.90 (m, H9,11, 4 H) 2.68 (t, J = 7 Hz, CH_2-propyl, 2 H), 1.88 (m, H10, 2 H), 1.52 (m, $CH_2CH_2CH_3$, 4 H), 1.02 (t, J = 7 Hz, CH_3, 3 H); ^{13}C NMR ($CDCl_3$), δ 158.8, 156.4, 151.8, 150.3, 147.8, 146.8, 146.0, 137.7, 135.8, 135.5, 133.1, 132.6, 129.6, 129.0, 127.6, 126.4, 126.3, 115.1, 112.8, 107.7, 31.2, 28.3, 27.3, 26.9, 26.6, 23.6, 23.3, 22.8, 13.9; IR (KBr) 3432 (w), 2950 (m), 2928 (m), 2226 (w), 1685 (s), 1654 (s), 1612 (s), 1542 (m), 1459 (m), 1388 (m), 1228 (m), 791 (w), 758 (w), 696 (w) cm^{-1}; FAB-MS m/z 473 (M + 1, 100 %). Anal. Calcd for $C_{31}H_{28}N_4O$•0.5 H_2O: C, 77.31; H, 6.07; N, 11.63; Found: C, 77.39;

H, 5.99; N, 11.60 %.

8-n-Butyl-3-cyano-1,6,7,9,10,11-hexahydrobenzo[b]pyrido[2,3-j][1,10]phenanthroline-2,12-dione (11). To a 100 mL round-bottomed flask were added 0.81 g (1.7 mmol) of crude **10**, 150 mL of CH_2Cl_2 and 100 mL of CH_3OH. The resulting solution was cooled to ca. -78 °C by means of a dry-ice/acetone bath and N_2 was bubbled through it for 5 m. A stream of O_2/O_3 was bubbled through the solution until the color changed from yellow to greenish-yellow. N_2 was bubbled through the solution for an additional 5 m, then 1.1 mL of dimethyl sulfide was added by syringe. The reaction mixture was allowed to warm to room temperature overnight, then concentrated by rotary evaporation. Ether was added to the resulting suspension and the product was collected by vacuum filtration as a yellow crystalline solid (0.60 g, 88 %) after drying at 61 °C (0.5 mm, 48 h), mp 290 °C (dec.). ^1H NMR ($CDCl_3$) δ 10.8 (bs, OH, 1 H), 8.19 (s, H5, 1 H), 7.87 (s, H4, 1 H), 3.04-3.10 (m, H6,7,11, 6 H), 2.77 (m, CH_2-propyl, H9, 4 H), 2.22 (m, H11, 2 H), 1.51 (m, $CH_2CH_2CH_3$, 4 H), 1.01 (t, J = 7 Hz, CH_3, 3 H); ^{13}C NMR ($CDCl_3$) δ 196.0, 158.9, 155.6, 150.3, 149.0, 148.7, 147.7, 146.1, 140.2, 137.3, 136.2, 129.3, 114.8, 113.4, 109.1, 39.2, 31.0, 28.5, 26.6, 26.3, 24.3, 23.1, 22.1, 13.8; IR (KBr) 3444 (m), 2956 (m), 2872 (m), 2225 (w), 1684 (s), 1616 (s), 1560 (m), 1458 (m), 1431 (m), 1389 (m), 1232 (m), 1171 (m), 973 (w), 791 (w) cm^{-1}; UV-visible (CH_3OH) λ_{max} (log ε) 232 (4.7), 298 (4.2), 386 (4.5), 404 nm (4.5); FAB-MS m/z 399 (M + 1, 100 %). Anal. Calcd for $C_{24}H_{22}N_4O_2 \cdot 1.5\ H_2O$: C, 67.75; H, 5.92; N, 13.17; Found: C, 67.79; H, 5.92; N, 13.13 %.

Complexation of Receptor 4 with Creatinine Hydrochloride. NMR Method. A mixture of 30 mg (0.2 mmol) of analytically pure creatinine hydrochloride and 2 mL of a 6 x 10^{-3} M solution of receptor **4** in $CDCl_3$ was shaken in a sealed centrifuge tube for 10 h. The supernatant solution was filtered through glass wool and the ^1H NMR spectrum was recorded (Figure 3). **UV-visible Method.** A 5.0 x 10^{-5} M solution of receptor **4** in 95:5 (v/v) $CHCl_3$/ethanol (3 mL) was mixed with excess creatinine hydrochloride. The mixture was stored overnight in a sealed container. The UV-visible spectrum of the supernatant solution was recorded with a Hewlett-Packard 8452A diode-array spectrophotometer and compared with that of the stock solution before exposure to creatinine hydrochloride (Figure 4).

Complexation of Receptor 9 with Creatinine. The following stock solutions were prepared in 70:30 (w/w) methanol/water using hplc grade methanol and deionized, distilled water: solution A, 7.2 mg of receptor **9** in 200 mL, 9.0 x 10^{-5} M; B, 2.033 g of creatinine in 100 mL, 0.180 M. All pH measurements were carried out under N_2 in a magnetically-stirred jacketed glass titration vessel, controlling temperature at 25.0 °C with water circulated from a Lauda constant temperature bath. A Corning 476540 semi-micro Ag/AgCl combination electrode was conditioned in deionized water for 2 weeks and calibrated at pH* values of 5.6 and 2.8 with the following buffers:[17] pH 5.6, 0.651 g (4.93 mmol) of dilithium succinate and 1.772 g (15.00 mmol) of succinic acid in 1.00 L of 70:30 (w/w) methanol/water; pH 2.8, 1.16 g (10.8 mmol) of ammonium hydrogen oxalate and 0.90 g (10.0 mmol) of oxalic acid in 1.00 L of 70:30 (w/w) methanol/water. In each creatinine complexation experiment 0.20-0.80 mL aliquots of stock solution B were mixed with 9.0 mL aliquots of stock solution A and the pH* of each resulting solution was adjusted to 4.3 by addition of HCl in 70:30 (w/w) methanol/water. Each solution was diluted to 10.0 mL, resulting in no further pH* change, and

the UV-visible spectra were recorded. The resulting spectra are shown in Figure 5 and the absorbances at 422 and 442 nm are plotted in Figure 6.

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