Metal triggered fluorescence sensing of citrate using a synthetic receptor

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A metal containing fluorescent chemosensor was designed, synthesized, and studied for the quantification of citrate in common beverages. The sensor consists of Cu(II) bound by a 1,10-phenanthroline ligand which is attached to a bis(aminoimidazolium) receptor (**5**). Receptor **5** was designed such that binding of the metal creates an additional binding site for citrate. This additional binding interaction was found to increase the metal and citrate binding constants in a cooperative manner, yielding a minimum 2.0 fold increase in the citrate binding constant and a minimum 2.0 fold increase in the Cu(II) binding constant. Further, **5** was designed so that binding of Cu(II) quenches a photo-excited state of the 1,10-phenanthroline fluorophore. Thus, addition of citrate to **5**–Cu(II) resulted in an increase of the fluorescence of the system. The nature of the fluorescence modulation upon citrate binding was probed using a model compound (**6**–Cu(II)). The data support an increase of electron density on the metal due to the donating ability of a carboxylate anion of citrate. In a sensing assay, the receptor is effective for measuring citrate concentrations in the micromolar range in highly competitive media. We believe this is the first demonstration of anion sensing in which the fluorescence emission is modulated due to a perturbation in the metal quenching effect upon analyte binding.

Introduction

The molecular recognition of anionic guest species by positively charged and neutral electron deficient hosts is currently receiving considerable interest.[1] The sensing and monitoring of anions can ultimately play a role in biomedical, biochemical, and environmental research.[2] Modulation of fluorescence is becoming a favored strategy due to its ease of analysis and high sensitivity.[3] Therefore, development of new strategies for the fluorescence based sensing of anions is an important goal. One strategy that has not been extensively studied involves binding anions directly to the fluorophore by way of a metal.

Previous examples of fluorophore sensors have relied mostly on photoinduced electron transfer (PET). In 1995, Tanaka demonstrated that the quenching of the emission of receptor **1** (see Fig. 1) with Ca(II) or Ba(II) bound in an appended crown ether was dependent on the type of counter ion present (perchlorate *versus* thiocyanate), and on the concentration of that counter ion.[4] The quenching which was observed with the thiocyanate anion but not the perchlorate anion was due to PET from the thiocyanate anion to the pyrido[1′,2′ : 1,2]imidazo-[4,5-*b*]pyrazine ring system.[5]

Using a similar strategy and in the same year, Fabbrizzi *et al.* synthesized a bis(dien) macrocycle bearing two picolinyl (pyridylmethyl) pendent arms (**2**) capable of binding two Cu(II) ions. Imidazole and molecules containing imidazole residues were found to bridge the two Cu(II) ions in an aqueous solution. Binding of these molecules was monitored *via* absorbance spectroscopy.[6] Building on this work a year later, Fabbrizzi and co-workers demonstrated that a transition metal–ligand interaction could be used for anion binding, and anion recognition could be signaled through fluorescence quenching.[7] Here, receptor **3** contains a Zn(II) ion. Quenching is observed only with carboxylates displaying distinctive electron donating or electron accepting tendencies due to a PET signal transduction mechanism.

In 1997, Shinkai and co-workers demonstrated two-point binding of sialic acid using receptor **4**, which features a Zn(II)–carboxylate coordination and a boronic acid–diol complex in the host–guest structure.[8] Once more, fluorescence modulation is the result of a PET mechanism in which the use of a nitrogen–boron interaction modulates the HOMO–LUMO interaction.[9] In all of these strategies the purpose of the metal was to coordinate an electron donor or acceptor close to a photo-excited fluorophore or simply to establish an additional binding site.

Our goal was to determine if the binding of an anion to a transition metal involved in fluorescence quenching could modulate fluorescence emission. We sought to turn the fluorescence on with addition of an analyte. With this strategy, an anion would no longer require electron donating or accepting properties to modulate the photo-excited fluorophore. Further, the use of a transition metal ion would not only quench the fluorophore, but also provide an additional binding site. Herein we report a fluorescence sensor based on this strategy.

Design criteria

Citric acid is a tricarboxylic acid that is trianionic at neutral pH and thus is highly water-soluble. Due to the hydrophilic and anionic nature of the guest, a receptor for citrate needs to be both water-soluble and possess the ability for strong binding of the anion in aqueous media. Nature's use of arginine to bind carboxylates suggests that guanidinium groups should be effective for charge pairing and hydrogen bonding to carboxylates in water.[10] Previous work from our group and others verifies this hypothesis.[11] For a guanidinium based receptor to become a sensor, a signaling device needs to be incorporated.

Phenanthroline was chosen as a signaling element for several reasons. First, phenanthroline is known to form stable

† The derivation of a general equation for the determination of equilibrium constants from absorption spectrophotometric data is available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b008694k/

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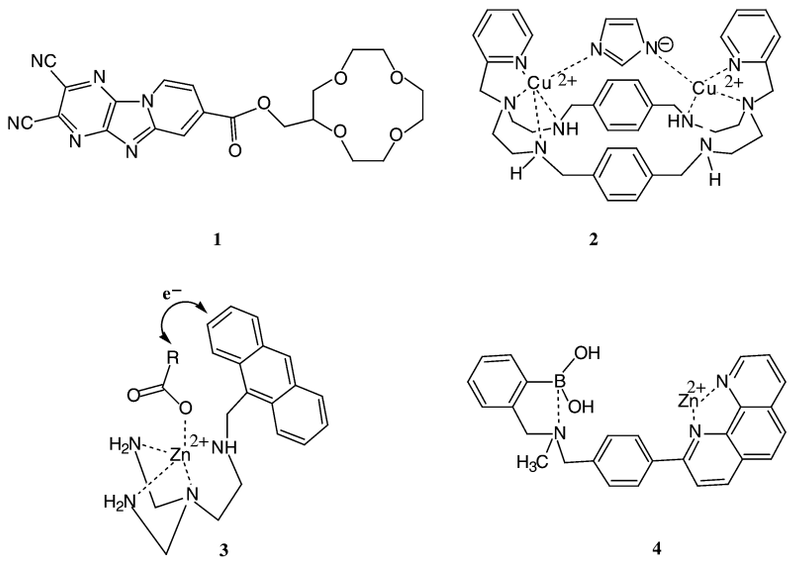
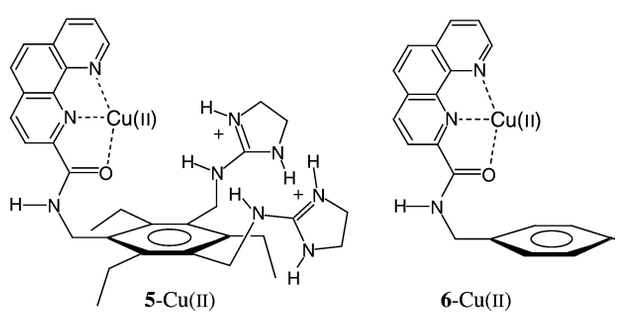


Fig. 1 Previous examples of fluorescent receptors utilizing energy transfer for signal transduction.

coordination complexes with transition metals.[12] Also, the pK_a of the conjugate acid of phenanthroline is relatively low (pK_a = 4.50).[12] In addition, the 2 and 9 positions are synthetically accessible, allowing substitution adjacent to the nitrogen. Further, phenanthroline can function as a fluorophore and/or a chromophore for analytical purposes.[13] Finally, upon metal binding, an additional binding site would be created for the trianionic guest.[14]

Once we identified a metal and a guanidinium functionality as the individual recognition elements for citrate, we needed a spacer to create a complementary receptor. Here an X-ray crystal structure of a triguanidinium receptor with propane-1,2,3-tricarboxylate suggested that a C_3 symmetric spacer derivatized with two guanidinium groups and a phenanthroline group would serve as a satisfactory receptor design.[15]

Following these criteria we designed receptor **5** where the metal can be chelated by the phenanthroline and the amide carbonyl.[16] The presence of Cu(II) in **5** creates an additional binding site for citrate since the citrate can act as a ligand to the Cu(II) ion. Conversely, binding of citrate to the guanidinium groups of **5** creates a third ligand for binding the metal. Hence, we predicted that the metal and the citrate would cooperatively increase each other's respective binding constants.[17]



5-Cu(II) **6**-Cu(II)

We also synthesized model compound **6** to probe the mechanism for fluorescence modulation upon citrate binding.[1] We wanted to be confident that the guanidinium groups were playing the roles designated. Compound **6** consists of a phenanthroline coupled only to a benzyl group. There are no aminoimidazolium groups to bind the carboxylates on the guest.

Results

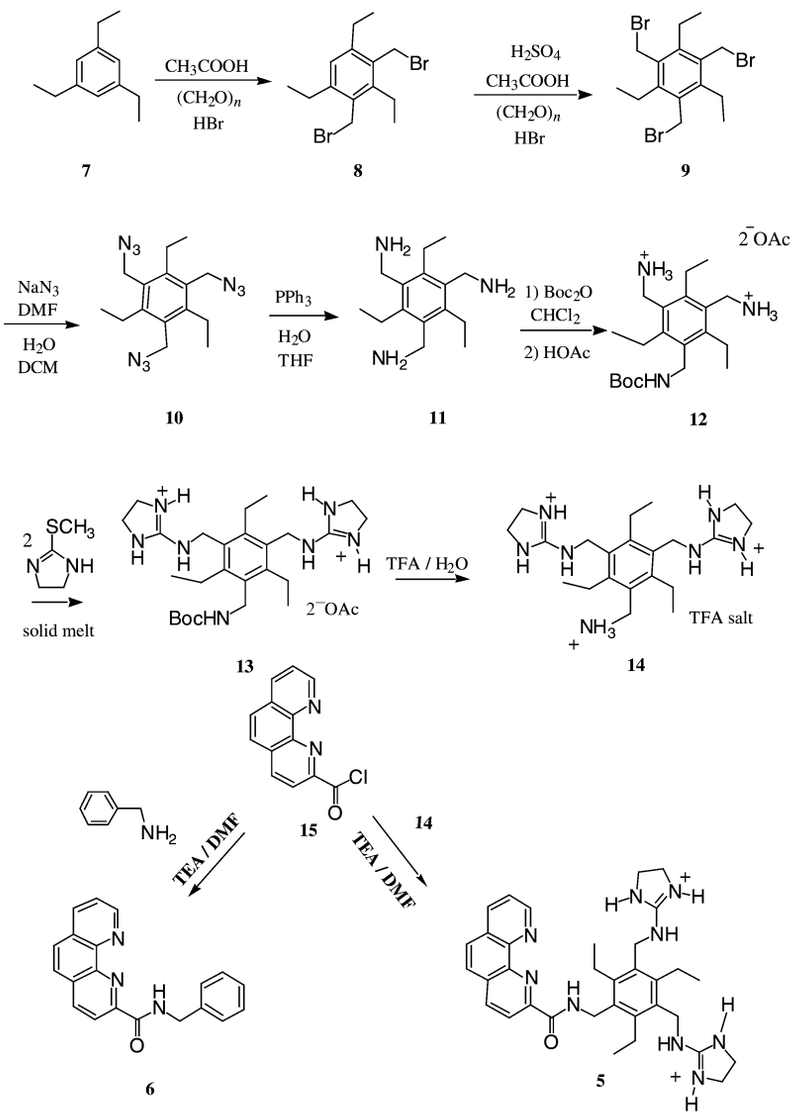
Synthesis

The synthetic routes for **5** and control compound **6** are shown in Scheme 1. Three bromomethyl groups are appended to 1,3,5-triethylbenzene (**7**) over two steps leading to 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene (**9**) in 52% yield. Formation of **9** over two consecutive steps is performed as single step formation of the compound afforded lower yields. Isolation of the 1,3-bis(bromomethyl)-2,4,6-triethylbenzene intermediate (**8**) is not necessary. Purification of **9** requires a lengthy chromatographic column using 2% dichloromethane–hexanes as eluant in order to separate **8** from **9**. To avoid this, a shorter column can be run to separate **8** and **9** from the other contents of the crude reaction mixture. These two compounds can then be used directly in the next reaction, following which the isolation of the 1,3,5-tris(azidomethyl)-2,4,6-triethylbenzene product (**10**) from the corresponding diazide product requires a much shorter column. Displacement of the bromides of a pure sample of **9** using sodium azide led to the formation of **10** in 89% yield.

The azides of **10** are then reduced to amines leading to 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene (**11**). This reaction is beneficial as the product can be isolated in 99% yield through washes. The reaction of **11**[18] with di-*tert*-butyl dicarbonate gave the mono-Boc protected compound **12** in a 45% yield after separation from di-Boc and tri-Boc protected compounds. Subsequently, the heterogeneous reaction of **12** with 2-(methylsulfanyl)dihydroimidazole in a sealed tube resulted in compound **13** in a 70% yield as the acetate salt after ion-exchange chromatography. Treatment of **13** with trifluoroacetic acid afforded the deprotected compound **14** in a 90% yield as the trifluoroacetate salt. Compound **15** was synthesized in four steps using a literature procedure.[19] The condensation of **14** and **15** gave **5** in an 85% yield as the acetate salt, which was converted to the chloride salt in a quantitative yield *via* ion-exchange chromatography. The condensation of **15** and benzylamine gave **6** in a 76% yield.[20]

Photophysical investigations; general considerations

Before starting the binding studies using our designed receptors, we first established experimental conditions for maintaining ionic strength and pH, and determined the proper concentrations to avoid collisional quenching of the emission. All experiments involved either **5**, **6**, **5**–$CuCl_2$, or **6**–$CuCl_2$. Each experiment was performed at 25 °C in a solution consisting of 85% methanol and 15% water (by volume) with HEPES buffer (1.0×10^{-4} M) at pH 7.4, along with NaCl (1.0×10^{-4} M) to maintain the ionic strength. Further, in all the studies described below, the host concentration was maintained at 1.37×10^{-5} M.



Scheme 1 Preparation of receptor **5** and control compound **6**.

At concentrations below 1.0×10^{-4} M, intermolecular or collisional quenching processes are not taking place.

UV–Visible absorption spectroscopy

UV–Vis spectroscopy was the first technique used to analyze the interactions of **5** and **6** with Cu(II). The λ_{max} for receptors **5** and **6** are the same (278 nm), with a very slight bathochromic shift observed (2 nm) upon complexation with $CuCl_2$. A small increase in the absorbance intensity is observed in the presence of $CuCl_2$, but it is not as dramatic a spectroscopic change as seen in the emission spectra. Little change in the UV–Vis absorption spectroscopy was found upon addition of citrate to a **5**–Cu(II) complex.

Fluorescence spectroscopy

Since absorption spectroscopy was not useful for detecting the interaction of citrate with metallated **5**, we turned to fluorescence. We first analyzed the interaction of the individual receptors **5** and **6** with $CuCl_2$. In these studies, the concentration of non-metallated receptors **5** and **6** were held constant and the emission of the phenanthroline ligand was monitored as a function of increasing $CuCl_2$. Upon excitation of **5** and **6** at 278.5 nm, a broad structureless emission centered at 365 nm was observed. Upon addition of $CuCl_2$, the intensity of the emission decreased (Fig. 2A and 2B). Hence, the binding of Cu(II) leads to quenching of the fluorescence of these receptors.

The experiments used to analyze the sensitivity of our sensor to citrate employed **5**–Cu(II) and **6**–Cu(II). Emission was monitored as a function of increasing citrate concentrations. The emission of the **5**–$CuCl_2$ complex increased dramatically with citrate, yet the emission of the **6**–$CuCl_2$ complex was relatively unaffected (Fig. 3A and 3B). Hence, only the Cu(II) complex of **5**, which contains guanidinium groups, acts as a citrate sensor.

In an additional study, the concentration of **5** was held constant and the emission was monitored as a function of increasing citrate. This was done to determine the necessity of the Cu(II) in the complex. The emission of **5** and **6** without added $CuCl_2$ was unaffected upon addition of citrate. Hence, the binding of Cu(II) triggers the sensitivity of **5** to citrate.

In our final study of the spectroscopic changes of **5** induced by Cu(II) and citrate, a solution of **5** and citrate was prepared. The emission was monitored as a function of increasing Cu(II) concentration. As anticipated, addition of $CuCl_2$ quenches the fluorescence, but the extent of quenching is less than without added citrate. At saturation, the quenching is 85% of that which is obtained when citrate is absent (compare Fig. 4 with Fig. 2A). Hence, the citrate mediates the extent to which Cu(II) quenches the phenanthroline fluorescence.

Discussion

Cyclic equilibria

The binding events between **5**, citrate, and Cu(II) define a cyclic

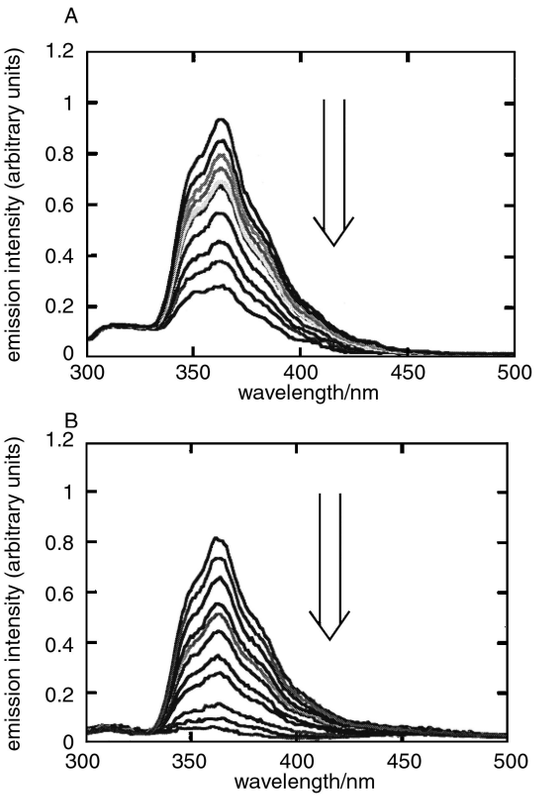


Fig. 2 A Influence on emission of receptor **5** with increasing $CuCl_2$ concentration. B Influence on emission of control compound **6**.

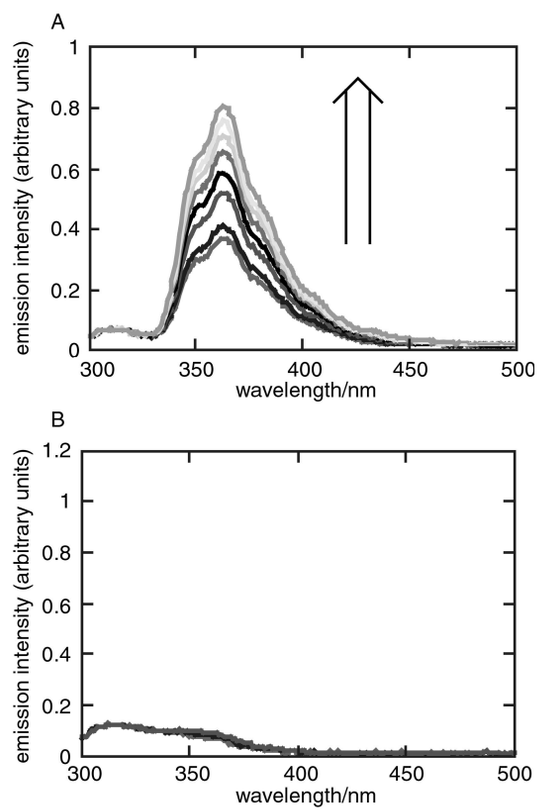


Fig. 3 A Influence on emission of the **5**–$CuCl_2$ complex with increasing citrate concentration. B Influence on emission of the compound **6**–$CuCl_2$ complex with increasing citrate concentration. The intensity of the emission signal was unaffected upon addition of citrate.

equilibrium expression (Scheme 2). Further, there is an equilibrium between Cu(II) and citrate that must be considered. The K_a for citrate and Cu(II) is 9×10^5 M^{-1} and the second K_a for a

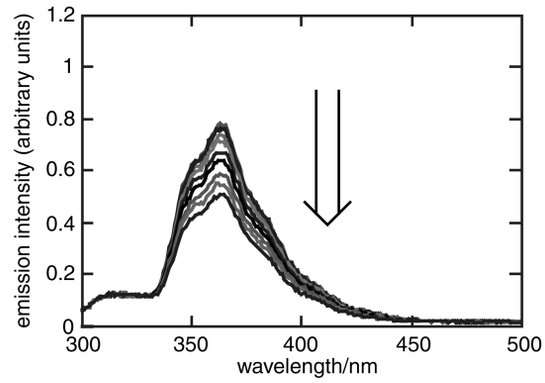


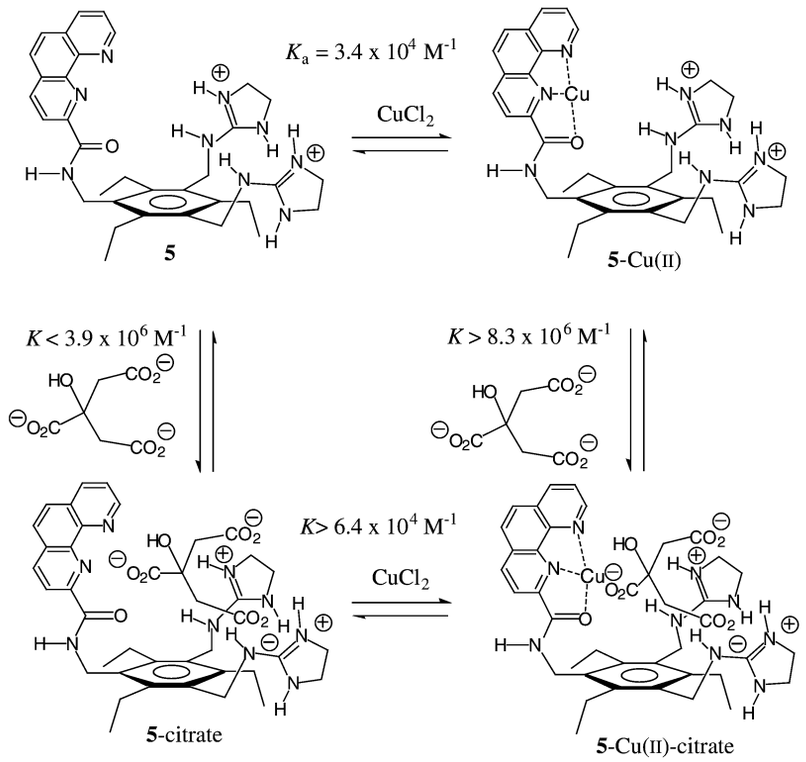
Fig. 4 Influence on emission of the **5**–citrate complex with increasing $CuCl_2$ concentration. The intensity of the emission decreased to a lesser extent than Fig. 2A upon addition of $CuCl_2$.

Cu(II) ion binding to a citrate–Cu(II) complex is 2×10^2 M^{-1}.[21] The photophysical data given in Fig. 2A, 3A, and 4 can be used to determine the association constants between **5** and Cu(II), **5**–Cu(II) and citrate, and **5**–citrate and Cu(II) respectively. Binding constants for the above complexes were determined using the general procedure of Rose and Drago[22] and a modified Rose and Drago equation derived using fluorescence intensities (see Supplementary Material). The case of solving the association constants between **5** and Cu(II) is simple since only one complex is forming, that between **5** and Cu(II) (Fig. 2A). Therefore, the binding constants can be determined directly. We find a K_a of 3.4×10^4 M^{-1} in an 85 : 15 methanol–water solution at pH 7.4.

Determining the exact binding constants between **5**–Cu(II) and citrate, and **5**–citrate and Cu(II) is more complicated. Now all the equilibria shown in Scheme 2 are present in solution. If one could start with equal concentrations of **5** and Cu(II) such that the **5**–Cu(II) complex was formed in a greater than 95% yield, titration with citrate would give a binding constant close to that for the **5**–Cu–citrate complex. Likewise, if we could titrate with Cu(II) starting with equal concentrations of **5** and citrate where the **5**–citrate complex was formed in a very high yield, we could determine the binding constant for the **5**–Cu–citrate complex. Unfortunately, at the concentrations required to form large mole fractions of the **5**–Cu(II) and **5**–citrate complexes, collisional quenching becomes dominant and therefore we cannot determine exact binding constants. Also, as mentioned previously, there is not enough change in the UV–Vis spectra to produce a reliable binding assay. Finally, NMR titration techniques are not applicable because the binding constants are too high for this technique to be practical, and Cu(II) is paramagnetic. Hence, we are forced to simply estimate the binding constants of the equilibria in the cycle shown in Scheme 2.

Under conditions where approximately 60 to 70% of the solution of **5** and Cu(II) or **5** and citrate were present as their respective complexes, we analyzed the data for titration with citrate and Cu(II) respectively. This resulted in minimum binding constants of 8.3×10^6 M^{-1} and 6.4×10^4 M^{-1} for **5**–Cu(II) with citrate and **5**–citrate with Cu(II) respectively (Fig. 3A and 4). These constants are lower limits on the actual binding constants because there is a competitive equilibrium between free **5** with the respective guests. Moreover, the binding between Cu(II) and citrate is also competitive.

Given that the association constant of **5** and Cu(II) is 3.4×10^4 M^{-1} and the association constant for **5**–citrate and Cu(II) is at least 6.4×10^4 M^{-1}, we conclude that there is at least a 2.0 fold increase in the binding of Cu(II) to **5** when citrate is also bound. In actuality, the increase in binding of Cu(II) due to the presence of citrate is likely to be much larger since under the experimental conditions used to measure the **5**–citrate and Cu(II) binding constant, much of the Cu(II) was undoubtedly



Scheme 2 The cyclic equilibria and equilibrium constants establish the cooperative binding of $CuCl_2$ and citrate to **5**.

associating with free citrate, thus competing for complexation with the **5**–citrate complex.

Using the minimum binding constant of 8.3×10^6 M^{-1} for **5**–Cu(II) with citrate, the minimum binding constant of 6.4×10^4 M^{-1} for **5**–citrate with Cu(II), and the association constant for **5** and Cu(II) for the cyclic equilibria, we were able to calculate a binding constant for **5** and citrate of 3.9×10^6 M^{-1}. This is now a maximum number since it is based partially upon two minimum numbers. However, since the association constant between **5** and citrate is at most 3.9×10^6 M^{-1}, and the association constant between **5**–Cu(II) and citrate is at least 8.3×10^6 M^{-1}, there is at least a factor of 2.0 increase in binding of citrate when Cu(II) is also bound. Again, it is likely that the cooperativity established between **5**, Cu(II), and citrate is significantly higher due to the competition between **5** and citrate for Cu(II) resulting from the equilibrium between Cu(II) and citrate.

Using the data in Fig. 2B, we determined the binding constant between **6** and Cu(II). This constant was found to be 8.9×10^5 M^{-1}. The $CuCl_2$ binding constant for **6** is about 30 times larger than **5** (8.9×10^5 and 3.4×10^4 M^{-1} respectively). This result was expected as the positively charged guanidinium groups of **5** should repel the Cu(II) ions.

Interpreting and extending the photophysical results

Addition of citrate to **5** produces a small response in the emission, yet in the presence of $CuCl_2$ the emission of **5** is very sensitive to the addition of citrate. The sensor must be in a quenched state (metal bound to the phenanthroline) for the citrate interaction to modulate the fluorescence. There are two possible modes of interaction between the **5**–Cu(II) complex and citrate which can result in a modulation of the fluorescence. First, and most obvious, is the possibility that the citrate is simply stripping the metal from the receptor. The phenanthroline would no longer be quenched and the fluorescence intensity would increase relative to the amount of citrate in solution. A second possibility is that the metal remains chelated to the phenanthroline and the citrate binds to the metal along with the guanidiniums of the receptor. Citrate acting as a ligand to the metal would increase the fluorescence since the metal would become more electron rich. Additionally, citrate complexation with Cu(II) would change the oxidation–reduction potential of the metal, thus changing the extent of electron transfer (quenching). The fluorescence intensity would increase until the metal–receptor complex is converted to a metal–receptor–citrate complex. The fact that the emission of **5** in the presence of citrate is quenched with Cu(II) to near 85% of that found with Cu(II) alone contradicts the hypothesis that citrate simply strips the metal from the complex. However, we wanted even more definitive proof that the citrate was not just stripping the metal from a complex with **5**. Therefore, we synthesized compound **6**.

The photophysical properties of **5** and **6** are almost identical with respect to quenching by Cu(II) (see Fig. 2A and 2B). The emission signal decreases dramatically with the addition of $CuCl_2$ for both molecules. In contrast, the photophysical properties are quite different with respect to the addition of citrate. Compound **6** in the presence of $CuCl_2$ is relatively insensitive to the addition of citrate, with little if any change in the emission signal (Fig. 3B). Apparently, there is little binding between citrate and the **6**–Cu(II) complex due to the absence of the two aminoimidazolium moieties. However, receptor **5** in the presence of one equivalent of $CuCl_2$ undergoes a large fluorescence enhancement with the addition of citrate (Fig. 3A). If the citrate were simply stripping the metal from the phenanthroline ligands, both **5** and **6** should behave similarly. This was not found.

In an extension of this experiment, we titrated a 1 : 1 : 1 ratio of **5**–Cu(II)–citrate with a $CuCl_2$ solution and observed only minor quenching even as the copper concentration approached twice that of the citrate. Evidently, a **5**–Cu(II) complex must remain intact in the presence of citrate. If the metal had been removed from the sensor through complexation with the citrate, the addition of $CuCl_2$ in concentrations exceeding that of citrate would have resulted in $CuCl_2$ forming a complex with **5**, thus quenching the emission. This was not observed. Therefore, given that only 85% of the maximum quenching is observed in the presence of citrate, that **5**–Cu(II) and **6**–Cu(II) behave differently upon addition of citrate, and that Cu(II) in excess of

Table 1 The results for the determination of citrate concentrations by **5**–Cu(II) and an NMR procedure are listed for two beverages and a known citrate concentration sample

Solution	Citrate concentration by weight/mmol dm^{-3}	Citrate concentration determined by NMR/mmol dm^{-3}	Citrate concentration determined by sensor **5**/mmol dm^{-3}
Power Aid	—	12.9	11.2
Gatorade	—	26.4	27.6
Citrate model	62.7	—	60.5

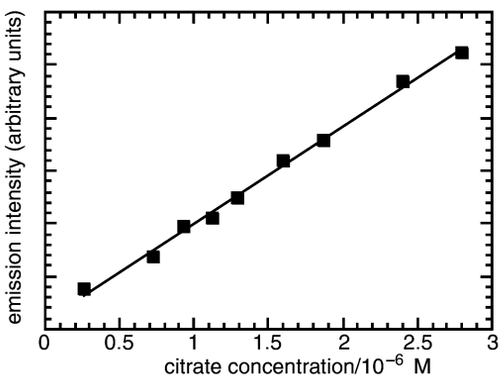


Fig. 5 The calibration curve for citrate concentration was created by incremental titration of the receptor–$CuCl_2$ complex.

citrate does not give increased quenching, we are confident that citrate does not simply strip the metal from **5**.

Hence, the increase in fluorescence of **5**–Cu(II) upon addition of citrate is due to a direct interaction between the fluorophore and the Cu(II). Moreover, since at the concentrations used in our studies citrate does not bind to **6**–Cu(II) but does bind to **5**–Cu(II), the guanidinium groups must be aiding the citrate recognition.

Collectively, these data demonstrate that the addition of a third entity, here a metal, can be used to both trigger the response of a receptor and provide an extra binding site for an analyte. Addition of Cu(II) establishes **5** in a quenched state and anion binding results in fluorescence modulation, which can be used to create a system capable of sensing an analyte that would not be possible without the trigger.

Calibration curve

Given that we had a sensor for citrate in hand, we wanted to determine if it was useful in a "real-life" application. As we have previously reported, we do this by analyzing beverages.[23] A calibration curve for citrate was created by incremental titration of a solution of 1.37×10^{-5} M **5**, 1.37×10^{-5} M $CuCl_2$, 1×10^{-4} M HEPES buffer at pH 7.4 and 1×10^{-4} M NaCl, with a citrate solution (1.37×10^{-4} M) which also contained the components listed. The intensity of emission was plotted *versus* citrate concentration (Fig. 5). Once the calibration curve was completed, a known volume of a beverage containing citric acid was added to a solution of **5**–Cu(II) identical to that which was used to create the calibration curve. In this study we limited our analysis to those beverages which present quite a competitive media, meaning high electrolyte content: sport drinks. A known citrate concentration was also investigated in this manner. To verify the concentration of citrate in the beverages the citric acid concentration was determined using a gravimetric NMR technique (see Table 1). In the NMR study, a known amount of beverage was diluted with water and lyophilized. The residue was dissolved in D_2O and lyophilized again. A known amount of this residue was placed into an NMR tube along with a known volume of D_2O. Using an NMR titration method with standard solutions of THF, and comparing the integration signals for the citrate *versus* the THF, the citrate concentration in the sample was determined. From this concentration, the original beverage concentration was determined.

The study demonstrated that **5**–Cu(II) is effective for a determination of the concentration of citrate at millimolar levels with less than a 10% error in repetitive measurements. The sensing system can easily be quantitative for micromolar levels of citrate, although for common beverages millimolar levels are sufficient.

Conclusions

It has long been known that metals can quench fluorescence through a number of processes.[24] Herein, we demonstrate that metal quenching can be modulated by an anionic guest in competitive media. Our evidence suggests a receptor–metal–citrate complex is responsible for this modulation. This study demonstrates that the guest need not possess electron donor or acceptor properties, but simply modulate the coupling between a metal quencher and a fluorophore. Currently, we are working on similar strategies that incorporate cooperative binding interactions that perturb concentrations of other bound and unbound signalling or triggering molecules, resulting in fluorescence modulations.

Experimental

A. General considerations

Instrumentation. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, CD_3CN, DMSO-d_6, CD_3OD, or D_2O used as purchased. NMR spectra were recorded on a General Electric QE-300, a Bruker AC-250, a Bruker AMX-500, a Varian Unity Plus (300 MHz), or a Varian Unity Inova (500 MHz) spectrometer. Melting points were measured on a Thomas Hoover capillary melting-point apparatus and were uncorrected. A SLM Aminco SPF 500 fluorescence spectrophotometer was used to record the fluorescence emission spectra and a Beckman DU-70 UV–Vis spectrometer was used for recording ultraviolet and visible absorption spectra.

Elemental analyses were performed by Atlantic Laboratories. Low-resolution and high-resolution mass spectra were measured with Finnigan TSQ70 and VG Analytical ZAB2-E instruments, respectively.

Materials. Preparative flash chromatography was performed on Scientific Adsorbents Incorporated Silica Gel 40 μm. Analytical thin layer chromatography was performed on precoated Silica Gel 60 F-254 plates. Cation exchange and reverse-phase (RP) liquid chromatography (LC) was performed on Sephadex-CM C-25 ion-exchange resin 40–120 μm and RP 18 C_{18}-modified silica gel 55–105 μm, respectively, using a Pharmacia LKB-FRAC-100 LC system. Solvents and reagents were purchased from Aldrich, Spectrum, Sigma, Lancaster, Fluka, and Mallinckrodt and used without purification, unless otherwise stated. Tetrahydrofuran and dimethoxyethane were distilled from sodium benzophenone ketyl. Dichloromethane and methanol were distilled from calcium hydride.

Compounds. Compound **15** was synthesized according to the method of Corey.[19]

B. Analytical studies

a. Binding studies. All fluorescence studies were carried out

in an 85% methanol–15% deionized water solution by volume. A typical experiment is described. Stock solutions were prepared for receptor **5**, citrate, control compound **6**, HEPES buffer, NaCl, and $CuCl_2$. These solutions were combined in a manner so that the initial concentration of the host was 1.37×10^{-5} M and the HEPES and NaCl concentrations were 1×10^{-4} M. A guest solution was prepared in exactly the same manner but with a guest concentration of 1.37×10^{-4} M. The pH of all solutions was adjusted to 7.4. Next, 2 mL of the host solution was placed in a quartz fluorescence cell and titrated with 10 μL additions of the guest. The fluorescence emission was recorded upon each addition. Excitation of the host solution at 278.5 nm gave emission centered near 365 nm. The emission intensity, host concentration, guest concentration, a cyclic equilibria analysis, and the general procedure of Rose and Drago[22] were used to determine binding constants (see Supplementary Material).

b. Calibration curve. Stock solutions of receptor, $CuCl_2$, and citrate were prepared as described above. 2 mL of the **5**–Cu(II) solution was placed in a quartz fluorescence cell and titrated with 3 μL additions of the citrate solution. The calibration curve for citrate was developed from these incremental titrations.

c. Determining citrate concentration. A typical experiment is described. A known volume of beverage was placed into a volumetric flask and brought to volume with an 85% methanol–15% deionized water solution by volume. In the case of the control citrate sample, a known amount of citrate in milligrams was placed into a volumetric flask and brought to volume with an 85% methanol–15% deionized water solution. At this point, a known volume of the test sample was placed into a fluorescence cell which contained 1 mL of a solution consisting of 1.37×10^{-5} M in **5**, 1.37×10^{-5} M $CuCl_2$, 1×10^{-4} M HEPES buffer at pH 7.4, and 1×10^{-4} M NaCl. The emission at 365 nm was taken and the calibration curve used to calculate citrate concentrations.

d. Determining citrate concentration. NMR method. A typical experiment is described. A known amount of beverage (5 mL) was placed into 200 mL of deionised water and lyophilized. The residue was dissolved in D_2O and lyophilized again. A known amount (50 mg) of this residue was placed into an NMR tube along with 1 mL of D_2O. Using an NMR titration method with standard solutions of THF, and comparing the integration signals for citrate *versus* THF, the citrate concentration in the sample was determined. From this concentration, the original beverage concentration was determined.

C. Synthesis

1,3-Bis(bromomethyl)-2,4,6-triethylbenzene (8). 1,3,5-Triethylbenzene (5 g, 31 mmol) and paraformaldehyde ‡ (3.1 g, 103 mmol) were combined and 18 mL of glacial acetic acid was added. This mixture was then heated to reflux at 130 °C, at which time 22 mL of a 33% hydrobromic acid–acetic acid solution was added. The reaction was allowed to reflux for 18 h and was quenched by pouring over 100 g of ice. The dark brown precipitate which resulted was then removed by filtration and the resulting mother liquor was neutralized using sodium carbonate and extracted with DCM. These extracts were combined with the precipitate and this solution was washed with sodium bicarbonate until bubbling upon addition ceased. The organic layer was then dried with magnesium sulfate, which was removed through filtration. Finally, solvent removal through rotary evaporation yielded a brown solid which was carried on to the next step without further purification.

‡ The IUPAC name for paraformaldehyde is poly(oxymethylene).

1,3,5-Tris(bromomethyl)-2,4,6-triethylbenzene (9). Unpurified **8** was combined with paraformaldehyde (4.1 g, 138 mmol), potassium bromide (16.4 g, 138 mmol) and 35 mL of glacial acetic acid. This solution was heated to reflux at 130 °C, at which time 12 mL sulfuric acid and 8 mL of acetic acid were added. The reaction was allowed to reflux for 10 hours and was poured over 100 g of ice. The resulting solution was brought to pH 5 through addition of sodium carbonate and then extracted with DCM. The extracts were washed with sodium bicarbonate until bubbling ceased and then were saturated with sodium chloride. Following drying with magnesium sulfate, the organic layer was filtered. Solvent removal with silica gel present led to a dried silica gel with the crude mixture adsorbed onto it. This mixture was then purified through column chromatography using 2% DCM–hexanes yielding **9** as a white solid (9.13 g, 52% over two steps): mp 159–160 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 4.58 (s, 6H), 2.95 (q, 6H), 1.34 (t, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) 144.96, 132.61, 28.54, 22.70, 15.56; CIHRMS *m/z* 437.9193 ($M^+ + H$, $C_{15}H_{21}Br_3$ calcd. found 437.9199).

1,3,5-Tris(azidomethyl)-2,4,6-triethylbenzene (10). 1,3,5-Tris(bromomethyl)-2,4,6-triethylbenzene (10 g, 23 mmol) was dissolved in 20 mL DCM and 50 mL DMF and heated to 80 °C with a condenser. A slurry of sodium azide (26.5 g, 41 mmol) in 20 mL water was formed and added through the top of the condenser. Following rinsing of the condenser with water, the reaction was stirred for 22 h. At this time, the DMF was removed by rotary evaporation and 100 mL of water and DCM were added to the crude mixture. The organic layer was extracted, followed by washing of the aqueous layer with DCM. The combined extracts were dried with sodium sulfate, filtered and the solvent was removed through rotary evaporation. The resulting crude oil was purified through column chromatography using 25% ethyl acetate–hexanes, yielding a white crystalline product (6.57 g, 89%): mp 61 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 4.49 (s, 6H), 2.85 (q, 6H), 1.24 (t, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) 144.93, 129.95, 47.89, 23.12 15.71; CIHRMS *m/z* 327.1920 ($M^+ + H$, $C_{15}H_{21}N_9$ calcd. found 327.1908).

1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene (11). 1,3,5-Tris(azidomethyl)-2,4,6-triethylbenzene (7 g, 21 mmol) and triphenylphosphine (37 g, 141 mmol) were combined in a 500 mL round-bottomed flask and 150 mL tetrahydrofuran and 3.9 g water were added. The solution was allowed to stir at room temperature overnight, during which time the solution bubbled vigorously. The THF was removed by rotary evaporation and the resulting solution was acidified with 200 mL of 50% HCl. The organic layer was then removed and extracted with 50% HCl. Combination of the aqueous layers was followed by addition of sodium hydroxide. The basic aqueous solution was then extracted with DCM. This extract was dried using sodium sulfate, filtered and the DCM was removed through rotary evaporation, yielding the fluffy white solid (5.29 g, 99%): mp 130–132 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 3.87 (s, 6H), 2.82 (q, 6H), 1.39 (s, 6H), 1.23 (t, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) 140.38, 137.43, 39.67, 22.58, 16.80; CIHRMS *m/z* 250.2282 ($M^+ + H$, $C_{15}H_{27}N_3$ calcd. found 250.2283).

1-{[(1,1-Dimethylethoxy)carbonyl]aminomethyl}-3,5-bis-(aminomethyl)-2,4,6-triethylbenzene dihydro diacetate (12). Di-*tert*-butyl dicarbonate (3.0 g, 13.8 mmol) dissolved in $CHCl_3$ (50 mL) was added dropwise to a solution of 1,3,5-tris-(aminomethyl)-2,4,6-triethylbenzene (5.7 g, 22.9 mmol) in $CHCl_3$ (200 mL). The solution turned from colorless to milky white during the addition and the mixture was then stirred under Ar for 8 h. Solvent removal under reduced pressure gave a white residue, which was purified by flash column chromatography with gradient elution of 2 to 10% NH_3 saturated CH_3OH in CH_2Cl_2 (v/v). The compound at $R_f = 0.3$ was

combined and concentrated. A small volume of 5% acetic acid (v/v) was added to the solid and the solution was lyophilized to give 3.45 g (43%) of dihydro diacetate salt **12** as a white solid: mp 124–126 °C; ^{1}H NMR (250 MHz, CD_3OD) 4.31 (s, 2H), 4.18 (s, 4H), 2.82 (q, 6H), 1.90 (s, 6H), 1.43 (s, 9H), 1.18 (t, 9H); ^{13}C NMR (62.8 MHz, CD_3OD) 180.0, 157.9, 146.9, 145.5, 133.9, 129.3, 80.7, 39.4, 37.6, 28.7, 24.1, 23.9, 23.7, 16.4, 16.4; CIHRMS *m*/*z* 350.281 ($M^+ + H$, $C_{20}H_{36}N_3O_2$ calcd. found 350.280).

1-{[(1,1-Dimethylethoxy)carbonyl]aminomethyl}-3,5-bis(4,5-dihydro-1*H*-imidazol-2-yl)aminomethyl]-2,4,6-triethylbenzene dihydro diacetate (13). Compound **12** (0.44 g, 0.95 mmol) and 2-methylthio-2-imidazoline (0.27 g, 2.40 mmol) were ground separately and then together with a mortar and pestle and pressed into a 2 mL conical vial. The vial was sealed and heated in an oil bath at 100 °C for 4 d. The mixture was cooled to rt. Dilute acetic acid (5% v/v) was added to dissolve and remove the mixture. Additional acetic acid was added (10 mL) and the mixture was lyophilized. The product was isolated by cation exchange on Sephadex with gradient elution of 100 mM to 1 M NH_4OAc. Fractions containing the last compound to be eluted were combined and the solution was lyophilized twice to give 0.204 g (36%) of dihydro diacetate salt **13** as a fluffy white solid: mp > 250 °C (dec.); ^{1}H NMR (250 MHz, CD_3OD) 4.39 (s, 4H), 4.31 (s, 2H), 3.75 (s, 8H), 2.76 (q, 6H), 1.83 (s, 6H), 1.43 (s, 9H), 1.18 (t, 9H); ^{13}C NMR (62.8 MHz, CD_3OD) 179.8, 161.1, 158.0, 146.1, 145.0, 134.1, 130.8, 80.3, 44.1, 42.3, 39.6, 28.8, 23.9, 16.5; CIHRMS *m*/*z* 604.381 ($M^+ - H$, $C_{30}H_{50}N_7O_6$ calcd. found 604.382).

1-(Aminomethyl)-3,5-bis[(4,5-dihydro-1*H*-imidazol-2-yl)-aminomethyl]-2,4,6-triethylbenzene trihydro trifluoroacetate salt (14). Compound **13** (0.61 g, 1.0 mmol) was dissolved in TFA (6.4 mL) and H_2O (3.5 mL) and stirred at rt for 4 h. The volatiles were removed under reduced pressure. More H_2O (10 mL) was added and the mixture was lyophilized to give 0.60 g (95%) of the trihydro tris(trifluoroacetate) salt **14** as a light tan solid: mp > 250 °C (dec.); ^{1}H NMR (500 MHz, CD_3OD) 4.47 (s, 4H), 4.27 (s, 2H), 3.77 (s, 8H), 2.74 (m, 6H), 1.18 (m, 9H); ^{13}C NMR (125 MHz, CD_3OD) 160.97, 146.70, 131.63, 129.75, 44.31, 42.03, 37.77, 24.19, 24.10, 16.31, 16.28; CIHRMS *m*/*z* 386.303 ($M^+ - H$, $C_{21}H_{36}N_7$ calcd. found 386.303).

1-[*N*-(1,10-Phenanthrolin-2-ylcarbonyl)aminomethyl]-3,5-bis-[(4,5-dihydro-1*H*-imidazol-2-yl)aminomethyl]-2,4,6-triethyl-benzene dihydro diacetate (5a). Compound **14** (1.0 g, 1.4 mmol) was dissolved in dry DMF (50 mL). At the same time, a solution of 0.3 g (1.2 mmol) of 2-carboxy-1,10-phenanthroline[19] in 40 mL of thionyl chloride was refluxed for 5 h. The thionyl chloride was then removed by evaporation and 20 mL of benzene was added. The benzene was evaporated to yield a yellow solid, which was used immediately. The solid was dissolved in 10 mL of dry DMF, 380 μL of dry TEA, and the solution was stirred. To this solution was added the trihydro tris(trifluoroacetate) salt solution dropwise. The reaction mixture was heated at reflux for 2 h and stirred overnight. The DMF was removed under reduced pressure, the residue was dissolved in 0.1 M HOAc and the mixture was lyophilized. The product was isolated by cation exchange on Sephadex with gradient elution of 100 mM to 1 M NH_4OAc. The final fractions were combined and the solution was lyophilized twice to give 0.84 g (1.2 mmol 85% yield) of dihydro diacetate salt **5a** as a white fluffy solid. ^{1}H NMR (500 MHz, CD_3OD) 8.98 (dd, 1H, J = 2.9 Hz), 8.52 (d, 1H, J = 8.35 Hz), 8.48 (dd, 2H, J = 2.95 Hz), 7.92 (dd, 2H, J = 7.2 Hz), 7.78 (dd, 1H, J = 3.6 Hz), 4.84 (s, 2H), 4.50 (s, 4H), 3.76 (s, 8H), 2.93 (q, 4H, J = 7.55 Hz), 2.78 (q, 2H, J = 7.55 Hz), 1.80 (s, 6H), 1.24 (m, 9H); ^{13}C {^{1}H} NMR (125 MHz, CD_3OD) 180.4, 166.3, 161.1, 150.7, 150.6, 146.6, 146.2, 145.4, 145.2, 139.1, 138.8, 133.8, 131.6, 131.1, 130.8, 129.3, 127.8, 125.1, 122.6, 44.2, 42.1, 39.2, 24.3, 24.1, 16.6, 16.5; CIHRMS *m*/*z* 592.351 ($M^+ - H$, $C_{34}H_{42}N_9O$ calcd. found 592.351).

1-[*N*-(1,10-Phenanthrolin-2-ylcarbonyl)aminomethyl]-3,5-bis-[(4,5-dihydro-1*H*-imidazol-2-yl)aminomethyl]-2,4,6-triethylbenz-ene dihydro dichloride (5). Compound **5a** (0.75 g, 1.1 mmol) was dissolved in H_2O (50 mL). The solution was eluted on a column of Amberlite IRA-400 resin (Cl^--form equilibrated at pH 7 with water). The resin was washed with more water (500 mL). The water was removed under reduced pressure, and the resulting solution was transferred to a smaller flask. The remaining water was removed by lyophilization to give 0.69 g (95%) of dihydro dichloride salt **5** as a light tan solid: mp > 250 °C (dec.); ^{1}H NMR (500 MHz, CD_3OD) 9.01 (dd, 1H, J = 2.9 Hz), 8.58 (d, 1H, J = 8.40 Hz), 8.48 (m, 2H), 7.97 (dd, 2H, J = 5.80 Hz), 7.78 (dd, 1H, J = 4.5 Hz), 4.86 (s, 2H), 4.53 (s, 4H), 3.78 (s, 8H), 2.92 (q, 4H, J = 7.50 Hz), 2.80 (q, 2H, J = 7.50 Hz), 1.25 (m, 9H); ^{13}C {^{1}H} NMR (125 MHz, CD_3OD) 166.4, 160.9, 150.8, 150.6, 146.8, 146.2, 145.5, 145.2, 139.2, 138.9, 133.8, 131.7, 131.2, 130.9, 129.4, 127.9, 125.2, 122.6, 44.2, 42.2, 39.3, 24.3, 24.2, 16.7, 16.5; CIHRMS *m*/*z* 592.351 ($M^+ - H$, $C_{34}H_{42}N_9O$ calcd. found 592.351).

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