A Fluorescent Sensor for 2,3-Bisphosphoglycerate Using a Europium Tetra-*N*-oxide Bis-bipyridine Complex for Both Binding and Signaling Purposes

Michael D. Best and Eric V. Anslyn*[a]

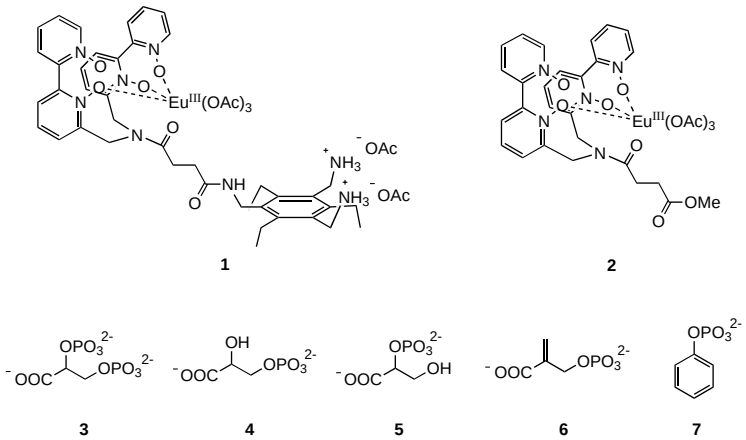
Abstract: Host **1** was designed and synthesized as a fluorescent sensor for 2,3-bisphosphoglycerate (BPG, **3**). The design features a tris-functionalized triethylbenzene core to preorganize binding groups. The three cationic moieties, a tetra-*N*-oxide bipyridine–europium complex and two ammonium groups, were included to complement the three anionic functionalities on the guest. Beyond acting as a binding site, the europium complex was used to signal binding of the guest through modification of the charge transfer emission. A 1:1 complex with BPG was determined in 50% methanol/acetonitrile with a K_a of $6.7 \times 10^5\ mol^{-1}$ by monitoring the reduction of the fluorescence signal upon guest addition. In the titration of related glycolytic intermediates lacking a second phosphate (**4**–**6**) into host **1**, 2:1 host to guest binding was observed. Similarly, control compound **2**, which lacks the ammonium groups, binds BPG and **4**–**6** in a 2:1 fashion. Also, phenylphosphate **7** binds to host **1** in a 1:1 stoichiometry with a K_a over three times less than **3**.

Keywords: bipyridines · europium · host–guest systems · lanthanide · receptors · sensors

Introduction

2,3-Bisphosphoglycerate (BPG, **3**) is a glycolytic intermediate formed in the interconversion between 3-phosphoglycerate (3-PG, **4**) and 2-phosphoglycerate (2-PG, **5**) by phosphoglycerate mutase.[1] BPG plays an important role in the regulation of oxygen transport by binding to hemoglobin and decreasing its affinity for oxygen.[2] As a result, inherited diseases exist in which abnormal concentrations of BPG lead to altered levels of oxygen transport.[3] Also, fetal hemoglobin has been found to exhibit higher oxygen affinity than adult hemoglobin due to a lower binding affinity towards BPG.[4] This has been attributed to a different N-terminal sequence on the β-chain of hemoglobin F.[5] BPG has been previously detected through the use of HPLC[6] and enzymatic assays.[7] In developing a fluorescent sensor for BPG, we sought to benefit from the sensitivity of fluorescent measurements, and to eventually develop a resin-bound sensor for incorporation into a multiple component sensing ensemble for the analysis of complex mixtures.[8]

Complexes between europium and bipyridine-derived macrocyclic structures have been the subject of extensive investigation, particularly due to their photophysical properties.[9] The excitation of the complex leads to multiple charge transfer emission signals with line-like properties. However, the fluorescence of the complex is often unstable in aqueous solution due to non-radiative decay into vibrational states of ligated waters. As a result, much work has focused on the development of cage-like bipyridine-based ligands to shield



1 **2** **3** **4** **5** **6** **7**

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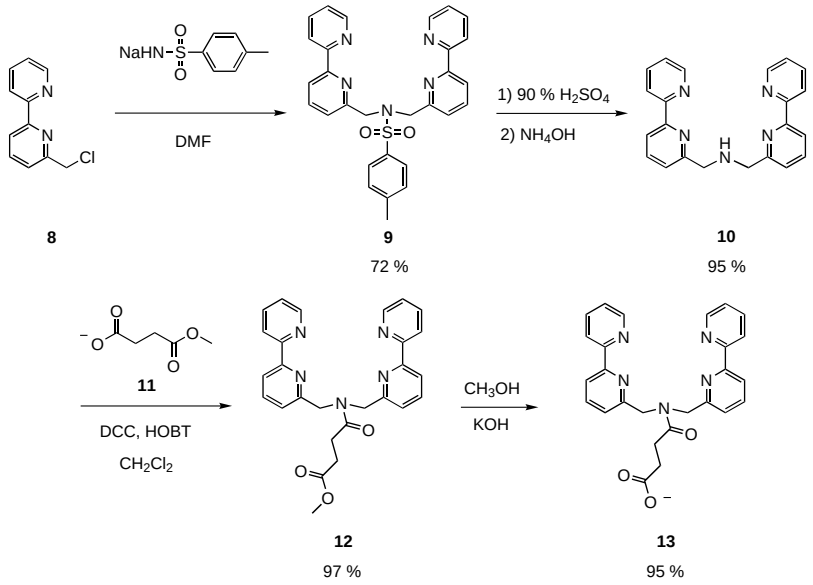
the europium from solvent and thus increase emission intensity.[10] Due to the increased affinity of N-oxidized bipyridine ligands towards europium[11], macrocyclic ligands for europium have also been developed containing these subunits.[12] Furthermore, polypyridine–europium complexes and other derivatives of lanthanides have been used for the fluorescent detection of anions and other analytes.[13]

The core design of host **1** is based on the tris-functionalized triethylbenzene scaffold. Due to steric repulsions between adjacent benzylic substituents, this structure preferably adopts a staggered conformation, placing the three binding groups towards the same face of the benzene ring, effectively forming a binding cavity.[14] We have used this scaffold to develop receptors for the detection of citrate[15], tartrate,[16] and gallate-like molecules[17] for the analysis of sports drinks, grape-derived beverages, and scotch whiskies, respectively. Other applications of this scaffold have been recently reviewed.[18] For host **1**, a tetra-*N*-oxide bipyridine group was included to form a stable complex with europium. The europium and ammonium groups were used to provide three cationic groups for binding of the three complementary anionic groups on the BPG.

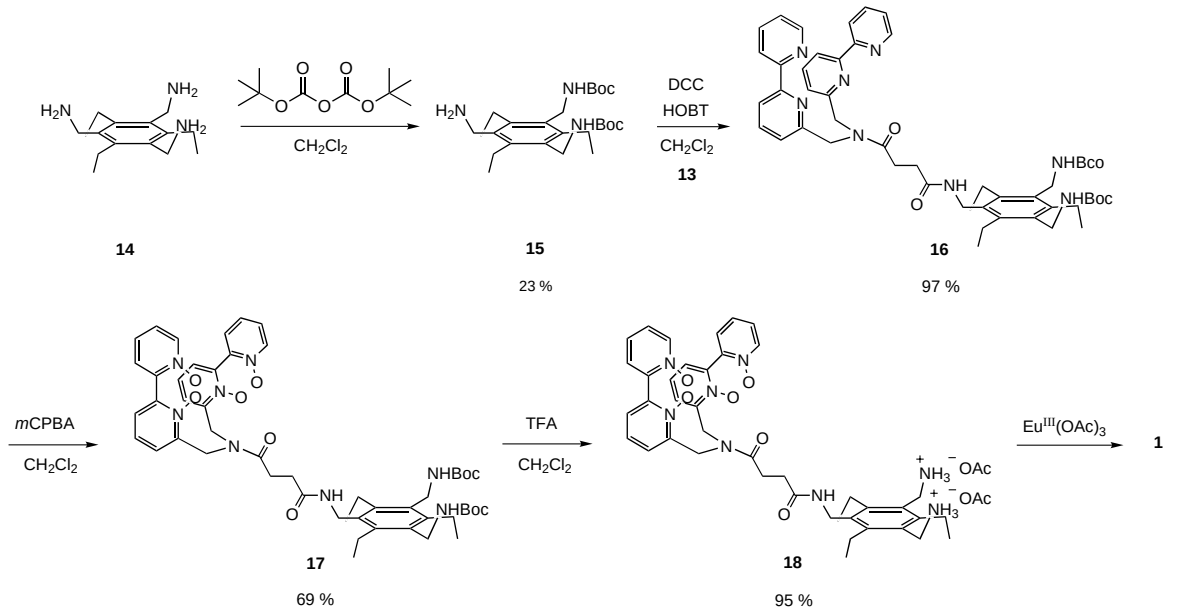


Scheme 1. Attachment of bipyridines to the succinate linker.

Results and Discussion

Synthesis:The synthetic scheme for host **1** begins with 6-chloromethyl-2,2′-bipyridine (**8**, Scheme 1).[19] This was allowed to react with sodium tosylamide leading to **9**.[20] Next, the tosyl group was removed with sulfuric acid in the formation of **10**. Following this, monomethylsuccinate (**11**) was coupled to the free amine of **10**, yielding **12**. Here, succinate was installed as a linker to increase the flexibility of the bipyridine ligands by distancing them from the relatively bulky triethylbenzene core. Succinate was specifically used as a linker such that all nitrogens were incorporated within amides to avoid their oxidation upon bipyridine N-oxidation later in the synthesis. Next, the methyl ester of **11** was hydrolyzed to afford **13**.

In the preparation of the triethylbenzene scaffold towards host **1**, two of the amines of tris(aminomethyl) structure **14**[15] were protected with *tert*-butyloxycarbonyl (Boc) groups, yielding **15** (Scheme 2). This was then coupled to carboxylate **13** using a standard peptide coupling protocol in the formation of **16**. Next, the bipyridines were oxidized using *m*-chloro-



Scheme 2. Synthesis of host **1**.

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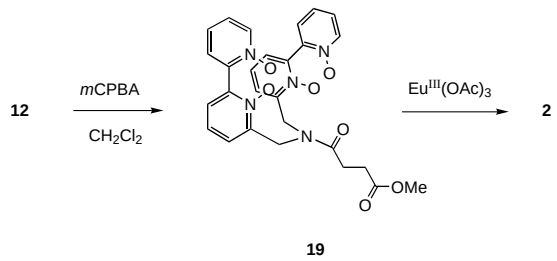
perbenzoic acid to afford compound **17**. Finally, deprotection of the Boc groups with trifluoroacetic acid followed by anion-exchange chromatography yielded ligand **18**. Europium complex **1** was then obtained by reaction of **18** with europium acetate hydrate and was characterized by the $[\mathbf{1}-\mathrm{OAc}]^+$ peaks at m/z 1017 and 1019 in the ESI-MS. Control compound **2**, which lacks the triethylbenzene scaffold and ammonium groups of **1**, was synthesized using the same strategy. Here, **12** was oxidized to **19**, which was then metallated to obtain model compound **2** (Scheme 3).



Scheme 3. Synthesis of model **2**.

Photophysical studies: The absorbance spectra of host **1** exhibited a λ_{max} of 260 nm for the complex. Upon exciting at this wavelength in methanolic solution, a fluorescence spectra was obtained which agreed with previous reports of the photophysical properties of similar bipyridine-*N*-oxide europium complexes.[12] This spectra features six line-like emission peaks at 577, 590, the largest peak at 610, 650, 690 and 699 nm. When the solvent was changed to greater than 5% water/methanol solutions, though, the fluorescence signal was completely quenched. However, with less than 5% water in the solvent, we were able to obtain reproducible results in the titration experiments.

Upon addition of 2,3-bisphosphoglycerate (**3**) to host **1** in methanol, a decrease in the fluorescent signals of the complex was observed. This was repeated with small percentages of water to ensure that hydrates on the guests were not the cause of the emission changes. In the titrations of **1** with BPG in methanol, the inflection point of mole ratio plots were at 0.5 equivalents of added guest and the data could not be fit to a 1:1 binding isotherm. This indicated that the host–guest complex which was formed in this solvent system consists of two hosts bound to one guest. Our hypothesis was that this stoichiometry might occur because the ammoniums of host **1** were not involved in complexation, and thus BPG binds the europiums of two separate hosts. As a result, we decided to decrease the polarity of the solvent to minimize the competition for hydrogen bonding and electrostatic interactions and thus drive potential interactions between BPG and the ammoniums of **1**.

Upon changing to 50% or less methanol in acetonitrile, the fluorescence decrease upon BPG addition to **1** fit a 1:1 binding curve. Also, in these solvents, the inflection of mol ratio plots consistently occurred at one equivalent of added guest, illustrating a discrete change in the binding mode. To obtain a **1**-BPG binding constant in 50% methanol/acetonitrile, several titrations were performed, the average of which yielded a K_a of $6.7\times10^5\ \mathrm{mol}^{-1}$ ($\pm$ 10%). A representative example of the fluorescence change upon BPG addition, along with the corresponding binding isotherm and 1:1 curve fit are illustrated in Figures 1 and 2, respectively.

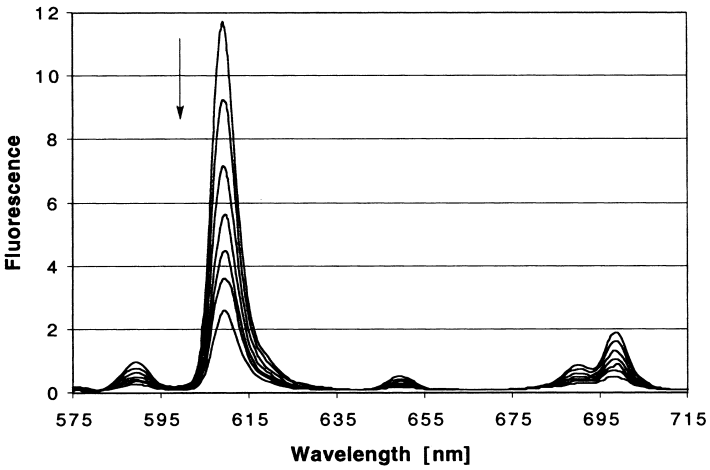


Figure 1. Fluorescence modulation upon introduction of BPG to **1**.

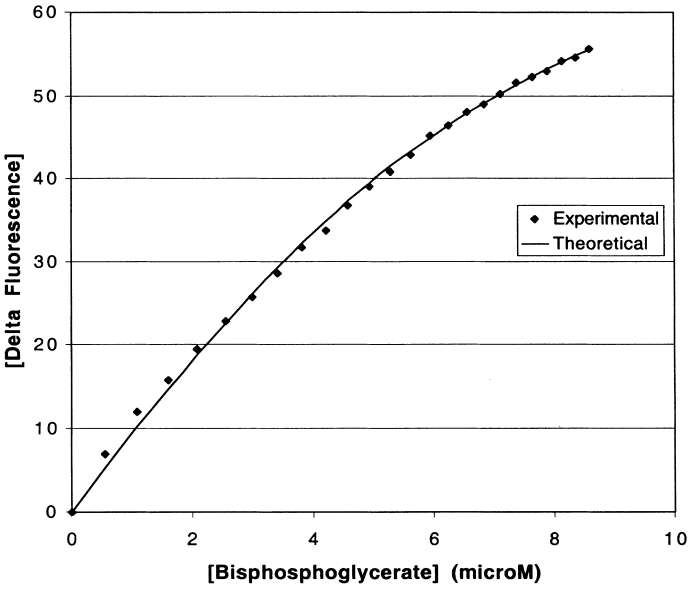


Figure 2. Binding isotherm and curve fit for **1**-BPG.

In addition to this fluorescence change, a very small shift in the excitation spectra of host **1** was observed upon addition of **3** (Figure 3). The presence of an isosbestic point in this shift was further evidence for the formation of a 1:1 complex in this solvent system. We also titrated BPG into control compound **2** in this solvent system, in which 2:1 host to guest binding was observed. This result indicates the importance of the ammonium groups of host **1** in the 1:1 complexation of BPG under these conditions as the removal of these groups causes the system to undergo this change in binding mode. Based on these results, a suggested binding mode for BPG in the complex (**20**) is presented in Scheme 4.

Next, we attempted titrations of other glycolysis intermediates: 3-phosphoglycerate (3-PG, **4**), 2-phosphoglycerate (2-PG, **5**), and phosphoenolpyruvate (PEP, **6**). Titrations of solutions of each of these compounds into solutions of **1** and **2**

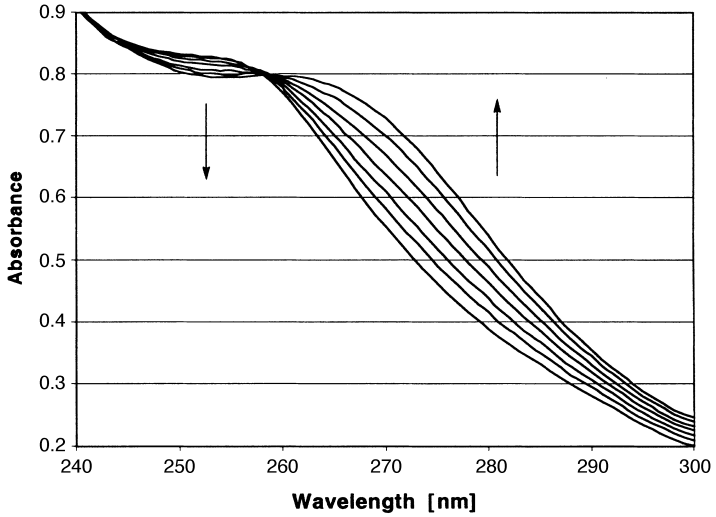
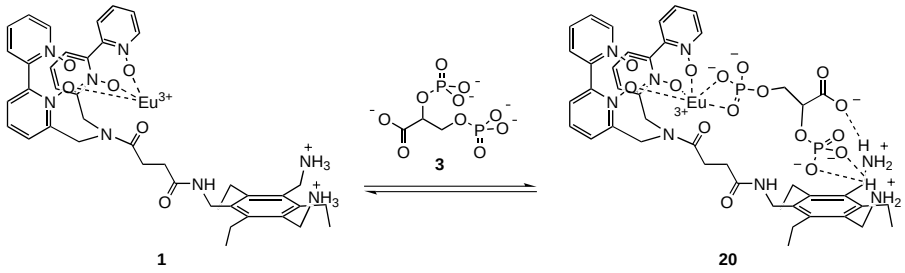


Figure 3. Shift in absorbance spectra of **1** with BPG addition.



Scheme 4. Hypothetical binding of BPG to host **1** based on titration evidence.

in solvent systems ranging from 10% methanol in acetonitrile to 100% methanol were performed. In all of these experiments, the inflection points in mol ratio plots pointed to the association of two hosts to one guest molecule. These results suggest that the extra phosphate on BPG is instrumental in forming a 1:1 complex with host **1**, the only guest from this group which forms a 1:1 complex. Any potential selectivity for BPG over **4** through **6** could not be directly quantified, though, due to the change in binding mode of the analytes.

The titration of phenylphosphate (**7**) was also performed. The data involving the change in emission upon introduction of **7** to host **1** in 50% methanol/acetonitrile could be fit to a 1:1 binding curve as was anticipated due to the presence of only one phosphate binding group. The K_a of this association was determined to be 2.0×10^5 mol^{-1} ($\pm$ 10%), a little more than three times less than that of the **1**-BPG complex. These data suggest a strong interaction of phosphates with the metal center of **1**, with only a small increase in affinity for BPG due to interactions with the ammonium groups. Results from the titrations of each guest in 50% methanol in acetonitrile are summarized in Table 1. Therefore the ammoniums in **1** influence the binding stoichiometry but hardly influence the binding affinity.

Conclusion

Host **1** forms a 1:1 complex with 2,3-bisphosphoglycerate in 50% methanol/acetonitrile, while the removal of the ammoniums to model **2** leads to 2:1 host to guest binding. This shows that the ammoniums of **1** play an important role in the binding of BPG in a 1:1 stoichiometry. Related glycolytic intermediates which lack the second phosphate undergo 2:1 host to guest binding, similar to **2**-BPG, most likely because they do not interact with the ammoniums of **1**. Phenylphosphate, with only one phosphate binding site, exhibits decreased affinity to the host, again probably due to the lack of interaction with the ammoniums of **1**. Thus, BPG showed the highest affinity towards **1** and was the only guest of the glycolytic intermediates to undergo 1:1 binding. However, problems with the host include the inability to detect binding in > 5% aqueous solutions due to quenching, and an inability to dramatically increase the affinity for BPG by changing the binding site functional groups.

Currently, we are attempting to develop other techniques for the detection of **1**-BPG binding in water, circumventing the quenching of the europium fluorescence in this solvent.

Table 1. Host–guest and model–guest titration results in 50% MeOH/MeCN.

Guest	K_a with host **1** ($\times 10^5$ M^{-1})	Result with host **2**
2,3-BPG (**3**)	6.7[a]	[b]
3-PG (**4**)	[b]	[b]
2-PG (**5**)	[b]	[b]
PEP (**6**)	[b]	[b]
phenylphosphate (**7**)	2.0[a]	[d]
acetate	[c]	[d]

[a] ±15%. [b] Undergoes 2 to 1 host-to-guest binding. [c] No change in emission spectra observed. [d] Not attempted.

Experimental Section

General: Methanol was heated under reflux over magnesium, tetrahydrofuan over sodium and benzophenone, and triethylamine and dichloromethane over calcium hydride, and distilled when noted. NMR tubes were dried in an oven at 125 °C for at least 24 hours prior to use. Products were placed under high vacuum overnight before spectra and masses were obtained. Starting materials were generally purchased from Aldrich. ^{1}H and ^{13}C NMR spectra were collected at 300 and 75 MHz, respectively, on a Varian Unity Plus spectrometer. High resolution mass spectra were recorded on a Finnigan VG analytical ZAB2-E spectrometer.

***N*,*N*-Bis-(6-(2,2′-bipyridyl)methyl)-*N*-tosylamine (9)**: 6-Chloromethyl-2,2′-bipyridine (**8**, 2.29 g, 11.1 mmol) was dissolved in *N*,*N*′-dimethylformamide (75 mL) in a flame dried 250 mL round-bottomed flask. Sodium tosylamide (1.08 g, 5.57 mmol) and potassium carbonate (2.31 g, 16.7 mmol) were then added and the reaction heated to 100 °C for 6 h. The solvent was removed through rotary evaporation and high vacuum pumping. Next, the crude was dissolved in dichloromethane (100 mL) and washed with water (3 × 100 mL). The organic layer was then dried with magnesium sulfate, filtered and the solvent removed by rotary evaporation and a high vacuum pump. The crude material was purified using column chromatography (silica gel, 70% ethyl acetate/hexanes). This yielded **9** as a white solid (2.04 g, 72%). M.p. 112–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.56$ (d, 2H, $J =$ 4.5 Hz), 8.10 (m, 4H), 7.62 (m, 6H), 7.31 (d, 2H, $J = 7.8$ Hz), 7.18 (m,

0947-6539/03/0901-0054 $ 20.00+.50/0 *Chem. Eur. J.* **2003**, *9*, No. 1

2H), 7.06 (d, 2H, $J = 8.1$ Hz), 4.73 (s, 4H), 2.19 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 155.24$, 155.09, 154.74, 148.43, 142.62, 136.85, 136.56, 136.06, 128.97, 126.63, 123.11, 122.09, 120.52, 118.96, 53.23, 20.79; HRMS-CI$^+$: m/z: calcd for $C_{29}H_{26}N_5O_2S$: 508.1807; found: 508.1808.

***N,N*-Bis-(6-(2,2′-bipyridyl)methyl)amine (10)**: Compound **9** (1.54 g, 3.0 mmol) was dissolved in 90% sulfuric acid (40 mL) in a 250 mL round-bottom flask. This was heated to 120 °C for 2 h under a condenser. After cooling to room temperature, the reaction was brought to pH 10 through addition of 1M sodium hydroxide, followed by concentrated ammonium hydroxide to yield a white precipitate. The solution was extracted with dichloromethane (3×200 mL), after which the extracts were combined, dried with magnesium sulfate, filtered and the solvent removed by rotary evaporation and a high vacuum pump. This yielded **10** as a brown oil (1.02 g, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.65$ (d, 2H, $J = 4.8$ Hz), 8.46 (d, 2H, $J = 8.1$ Hz), 8.25 (d, 2H, $J = 7.8$ Hz), 7.87–7.69 (m, 4H), 7.35 (d, 2H, $J = 7.5$ Hz), 7.27 (t, 2H, $J = 6.3$ Hz), 4.09 (s, 4H), 3.22 (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 158.81$, 155.96, 155.23, 148.82, 137.03, 136.48, 123.33, 121.99, 120.92, 118.94, 54.40; HRMS-CI$^+$: m/z: calcd for $C_{22}H_{20}N_5$: 354.1719; found: 354.1712.

Methyl-3-[*N,N*-bis-(6-(2,2′-bipyridyl)methyl)carbamoyl] propanoate (12): Dicyclohexylcarbodiimide (527 mg, 2.55 mmol), hydroxybenzotriazole (345 mg, 2.55 mmol) and monomethyl succinate (337 mg, 2.55 mmol) were dissolved in 5 mL of distilled dichloromethane in a flame dried 100 mL round-bottom flask. This was allowed to stir at 0 °C under an argon balloon for 30 minutes. Compound **11** (752 mg, 2.13 mmol) was separately dissolved in 20 mL of distilled dichloromethane and then added to the reaction flask. The reaction mixture was allowed to stir at 0 °C for 30 minutes and then at room temperature for 6 h. Next, the crude material was directly added to a silica gel column and purified using gradient elution from 1 to 3% methanol/dichloromethane. This afforded **12** as a white solid (963 mg, 97%). M.p. 88–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.59$ (t, 2H, $J = 4.2$ Hz), 8.18–8.36 (m, 4H), 7.71 (m, 4H), 7.30–7.17 (m, 4H), 4.78 (s, 2H), 4.76 (s, 2H), 3.53 (s, 3H), 2.79–2.62 (m, 4H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 173.35$, 172.28, 156.56, 155.92, 155.82, 155.61, 155.49, 155.21, 148.92, 148.87, 137.59, 137.40, 136.76, 136.57, 123.68, 123.43, 122.18, 121.00, 120.91, 119.52, 119.28, 52.99, 51.63, 51.53, 29.07, 28.08; HRMS-CI$^+$: m/z: calcd for $C_{27}H_{26}N_5O_3$: 468.2036; found: 468.2032.

3-[*N,N*-Bis-(6-(2,2′-bipyridyl)methyl)carbamoyl] propanoate (13): Compound **12** (765 mg, 1.64 mmol) was dissolved in methanol (25 mL) and 1M KOH (25 mL) in a 250 mL round-bottomed flask. The reaction was heated to 90 °C for 5 h. The solvent was then removed through rotary evaporation and a high vacuum pump to afford an orange oil. The crude material was then dissolved in chloroform, dried with magnesium sulfate, filtered and the solvent removed. Purification was performed using column chromatography on silica gel with 20% ammonia sat. methanol/dichloromethane as eluant, yielding **13** as a brown solid (740 mg, 95%). M.p. 66–68 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.48$ (s, 2H), 8.21 (d, 1H, $J = 8.1$ Hz), 8.06 (t, 2H, $J = 8.1$ Hz), 7.93 (d, 2H, $J = 8.1$ Hz), 7.63–7.47 (m, 4H), 7.09–7.07 (m, 4H), 4.67 (s, 2H), 4.62 (s, 2H), 2.68 (s, 2H), 2.43 (s, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 177.55$, 173.83, 156.59, 155.90, 155.80, 155.57, 155.23, 149.00, 148.96, 137.77, 137.64, 137.05, 136.86, 123.83, 123.60, 122.39, 121.24, 121.18, 119.71, 119.53, 53.16, 51.69, 50.54, 30.68, 28.78; HRMS-CI$^+$: m/z: calcd for $C_{26}H_{24}N_5O_3$: 454.1880; found: 454.1871.

1,3-Bis[*N*-((*tert*-butoxy)carbonyl)aminomethyl]-5-(aminomethyl)-2,4,6-triethylbenzene (15): 1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene (**14**, 8.85 g, 35.5 mmol) was dissolved in distilled dichloromethane (300 mL) in a 1 L round-bottomed flask and was placed under nitrogen. Boc_2O (8.53 g, 39.1 mmol) was dissolved in distilled dichloromethane (150 mL) and placed under nitrogen in an addition funnel on the reaction flask. This solution was dropped into the reaction slowly over 2 h. Upon addition, the solution turned cloudy. The solvent was removed by rotary evaporation after 21 h, and the resulting milky white solid was purified by column chromatography (silica gel, gradiant elution from 1% to 30% ammonia sat. methanol/dichloromethane). TLC analysis of the fractions was performed using ninhydrin spray, which stained the otherwise unidentifiable spots of the amine products. The first band yielded the tris-Boc protected compound as a white solid after evaporation (1.47 g, 8%). The next band was the bis-Boc protected amine (**15**), a white fluffy solid (3.62 g, 23%), and the third band afforded the mono-Boc protected product also as a white, fluffy solid (2.65 g, 21%). The unreacted amine was then removed using 30% ammonia sat. methanol/dichloromethane, and obtained as a yellow solid (3.52 g, 40%). M.p. 142–144 °C; ^{1}H NMR (300 MHz, $CDCl_3$) (free based compound): $\delta = 4.48$ (s, 2H), 4.26 (s, 4H), 3.79 (s, 2H), 2.68 (q, 6H, $J = 7.5$ Hz), 1.37 (s, 18H), 1.13 (t, 9H, $J = 7.5$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 155.16$, 142.38, 142.28, 136.91, 131.96, 78.97, 39.09, 38.49, 28.11, 22.55, 22.40, 16.36, 16.23; HRMS-CI$^+$: m/z: calcd for $C_{25}H_{44}N_3O_4$: 450.3332; found: 450.3332.

1-[(3-(*N,N*-Bis-(6-(2,2′-bipyridyl)methyl)carbamoyl)propamoyl)aminomethyl]-3,5-bis[*N*-((*tert*-butoxy)carbonyl)aminomethyl]-2,4,6-triethylbenzene (16): Compound **15** was free based by addition of dichloromethane and concentrated ammonium hydroxide, extraction of the dichloromethane, washing of the aqueous layer, combination of the organic layers, drying and solvent removal. After placement on the high vacuum pump overnight, this compound (483 mg, 1.08 mmol) was dissolved in distilled dichloromethane (20 mL) in a 100 mL round-bottomed flask. The flask was then cooled to 0 °C and placed under argon. To this was added **13** (493 mg, 1.08 mmol), dicyclohexylcarbodiimide (270 mg, 1.29 mmol) and hydroxybenzotriazole (177 mg, 1.29 mmol). The reaction was then warmed to room temperature and stirred for 14 hours. Solvent removal yielded a brown oil, which was purified by column chromatography over silica gel with gradient elution from 0.5 to 1.5% methanol/dichloromethane. This yielded **16** as a yellow solid (965 mg, 97%). M.p. 108–112 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.55$ (d, 2H, $J = 4.8$ Hz), 8.29–8.13 (m, 4H), 7.75–7.61 (m, 4H), 7.25–7.14 (m, 4H), 6.25 (s, 1H), 4.83 (s, 2H), 4.76 (s, 2H), 4.62 (s, 2H), 4.33 (s, 2H), 4.21 (s, 4H), 2.97 (t, 2H, $J = 6.0$ Hz), 2.69–2.55 (m, 8H), 1.08 (s, 18H), 1.05–1.03 (m, 9H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 173.06, 171.95, 157.11, 156.42, 155.87, 155.63, 155.40, 155.23, 148.85, 143.49, 137.64, 137.41, 136.85, 136.68, 132.06, 131.67, 123.68, 123.50, 121.96, 121.11, 121.03, 120.99, 119.66, 119.39, 79.19, 33.65, 31.26, 29.44, 28.92, 53.27, 53.03, 51.48, 38.53, 37.84, 28.20, 22.63, 16.17; HRMS-CI$^+$: m/z: calcd for $C_{51}H_{65}N_8O_6$: 885.5027; found: 885.5028.

1-[(3-(*N,N*-Bis-(6-(2,2′-bipyridyl-1,1′-dioxide)methyl)carbamoyl)propamoyl)aminomethyl]-3,5-bis-[*N*-((*tert*-butoxy)carbonyl)aminomethyl]-2,4,6-triethylbenzene (17): Compound **16** (167 mg, 188 μmol) was dissolved in chloroform (2 mL) in a 50 mL round-bottomed flask, which was then placed at 0° C. *m*-Chloroperbenzoic acid (233 mg, 943 μmol) was dissolved in chloroform (3 mL) and added to the reaction mixture. The reaction flask was then brought to room temperature with stirring for 3 h. A second portion of *m*CPBA (233 mg, 43 μmol) was dissolved in chloroform (3 mL) and added to the reaction, followed by stirring for 17 more hours. Following solvent removal by rotary evaporation, the crude mixture was purified through column chromatography over silica gel. Here, methanol (1 L) was passed through the column to elute all biproducts. Then, ammonia saturated methanol (1 L) was used to remove **17** (123 mg, 69%). M.p. 173–175 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.26$ (s, 2H), 7.50–7.30 (m, 12H), 5.87 (s, 1H), 4.99 (s, 2H), 4.86 (s, 2H), 4.54 (s, 2H), 4.40 (s, 2H), 4.31 (s, 4H), 2.87 (t, 2H, $J = 6.0$ Hz), 2.64 (m, 6H), 2.48 (t, 2H, $J = 6.0$ Hz), 1.39 (s, 18H), 1.11 (m, 9H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 173.56$, 171.59, 155.28, 147.56, 146.84, 143.67, 142.83, 142.74, 142.52, 142.39, 139.81, 132.32, 131.88, 128.12, 127.03, 126.57, 126.41, 126.28, 125.21, 125.02, 124.95, 124.83, 48.52, 45.76, 38.64, 37.96, 30.73, 30.19, 28.29, 25.03, 22.78, 16.34; HRMS-CI$^+$: m/z: calcd for $C_{51}H_{65}N_8O_{10}$: 949.4824; found: 949.4827.

1-[(3-(*N,N*-Bis-(6-(2,2′-bipyridyl-1,1′-dioxide)methyl)carbamoyl)propamoyl)aminomethyl]-3,5-bis(aminomethyl)-2,4,6-triethylbenzene bis(acetic acid) salt (18): Compound **17** (140 mg, 148 μmol) was dissolved in distilled dichloromethane (2 mL) and trifluoroacetic acid (2 mL). The reaction was stirred for 3 h, at which time solvent removal yielded an orange oil. The crude was then run down an Amberlite IRA-400 (Cl^-) anion exchange column which had been treated with ammonium acetate. The resulting salt was lyophilized and then redissolved in water and lyophilized twice more to yield **18** as a brown solid (122 mg, 95%). M.p. >200 °C; ^{1}H NMR (300 MHz, CD_3OD): $\delta = 8.36$ (s, 2H), 7.62–7.48 (m, 13H), 4.97 (s, 2H), 4.74 (s, 2H), 4.35 (s, 2H), 3.98 (s, 4H), 3.21 (s, 4H), 2.91–2.44 (m, 6H), 1.80 (s, 6H), 1.07 (m, 9H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 176.07$, 174.37, 161.35, 160.83, 160.31, 159.79, 150.52, 149.35, 147.56, 145.95, 144.43, 144.25, 143.92, 141.73, 141.56, 134.36, 133.90, 132.81, 131.68, 130.97, 130.64, 129.68, 129.56, 129.04, 127.84, 122.52, 118.72, 114.92, 111.11, 38.59, 37.84, 31.35, 29.25, 24.18, 16.29; HRMS-CI$^+$: m/z: calcd for $C_{41}H_{49}N_8O_6$: 749.3775; found: 749.3784.

1-[(3-(*N,N*-Bis-(6-(2,2′-bipyridyl-1,1′-dioxide)methyl)carbamoyl)propamoyl)aminomethyl]-3,5-bis(aminomethyl)-2,4,6-triethylbenzene bis(acetic acid) europium(III) trisacetate (1): Compound **18** (26.7 mg, 30.8 μmol)

  0947-6539/03/0901-0055 $ 20.00+.50/0

was placed in a 50 mL round-bottomed flask and dissolved in spectroscopic grade methanol (5 mL). Europium acetate hexahydrate (13.4 mg, 30.8 μmol) was then added and the solution heated to 60° C under a condenser for 3 h. Following the reaction, the mixture was diluted into a stock solution and used for studies without further purification.

Methyl-3-[*N*,*N*-bis-(6-(2,2′-bipyridyl-1,1′-dioxide)methyl)carbamoyl]propanoate (19): Structure **12** (183 mg, 391 μmol) was dissolved in chloroform (2 mL) in a 100 mL round-bottomed flask, which was placed at 0° C. *m*-Chloroperbenzoic acid (482 mg, 1.96 mmol) was then dissolved in chloroform (3 mL) and added to the reaction flask through a syringe. The reaction was stirred at 0° C for 15 min and then at room temperature for 4 h. At this point, the reaction was returned to 0° C, and another portion of *m*CPBA (482 mg, 1.96 mmol) dissolved in chloroform (5 mL) was added. After 15 minutes, the reaction was again allowed to warm to room temperature, and was then allowed to stir for 15 h. Next, the reaction was directly loaded onto a silica gel column. First, the *m*-CPBA and resultant *meta*-chlorobenzoic acid were removed using 100% methanol as eluant. This was followed by the removal of the methyl-3-(*N*,*N*-bis-(6-(2,2′-bipyridyl-1,1′-dioxide)carbamoyl))propanoate using 25% ammonia saturated methanol/dichloromethane as eluant. The product **19** was obtained as a light yellow solid (208 mg, 97%). M.p. 115–117° C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 8.27 (t, 2H, $J = 6.0$ Hz), 7.50–7.28 (m, 12H), 4.97 (s, 2H), 4.87 (s, 2H), 3.65 (s, 3H), 2.82 (t, 2H, $J = 6.0$ Hz), 2.67 (t, 2H, 6.0 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 173.35, 173.10, 147.57, 146.92, 142.87, 142.48, 139.96, 139.90, 128.18, 127.06, 126.61, 126.45, 125.53, 124.95, 124.91, 124.81, 51.75, 48.55, 45.71, 28.84, 27.88; HRMS-CI$^+$: *m*/*z*: calcd for $C_{27}H_{26}N_5O_7$: 532.1832; found: 532.1840.

Methyl-3-[(*N*,*N*-bis-(6-(2,2′-bipyridyl-1,1′-dioxide)methyl)carbamoyl)propanoate] europium(III) trisacetate (2): Molecule **19** (26.0 mg, 48.7 μmol) was dissolved in spectroscopic grade methanol (5 mL) in a 50 mL round-bottomed flask. To this was added europium acetate hexahydrate (21.4 mg, 48.7 μmol). The reaction was heated to 70° C under a condenser. Following this, the solution was diluted into a stock solution and used for studies without further purification. MS-ESI$^+$ [**2** – OAc]$^+$: found: 800, 802; MS-FAB$^+$ [**2** – OAc]$^+$: found: 800, 802; HRMS-FAB$^+$: *m*/*z*: calcd for $C_{31}H_{31}N_5O_{11}Eu$: 802.1233; found: 802.1223.

Titrations of guests into host 1 and model 2: Two solutions were freshly made for each titration, the cuvette solution consisting of only host, and the titrant having the same concentration of host with a concentration of guest four to five times larger. The cuvette solution was formed by adding an unbuffered host stock solution (100 μL) and diluting with 5 mM tris-buffer (4.9 mL), which was adjusted to pH 7.4 through methoxide addition, and spectroscopic grade acetonitrile (5 mL). Thus, the final solution contained 5.52 nM host and 2.45 mM buffer in 50% methanol/acetonitrile. The guest solution contained 100 μL of the host as well, plus, in the case of BPG, 700 μL of guest buffered to pH 7.4 with 5 mM tris in methanol, 4.3 mL of 5 mM Tris-buffered methanol and acetonitrile (5 mL). The resulting guest solution contained 5.52 nM host, 22.9 nM BPG and 2.45 mM tris buffer in 50% methanol/acetonitrile. Titrations were performed by adding the host solution (2 mL) to a quartz cuvette and adding guest solution (50 μL) for each point, leading to a first addition [BPG]/[host] ratio of around 0.1. Titrations were carried out to around three equivalents of guest. Binding affinities were calculated through curve fitting of the changes in fluorescence. This was performed through iterative manipulation of the binding constant and change in molar absorptivity until the best fit of the data with the theoretical curve was recorded.

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