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Fluorescent bis-cyclen tweezer receptors for inositol (1,4,5)-trisphosphate

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ABSTRACT

Herein, we report the development of two fluorescent sensors for inositol 1,4,5-trisphosphate ($InsP_3$), containing either two free cyclen or zinc(II)/cyclen groups preorganized into a binding cleft. Preorganization was achieved using a rigid acridine backbone, which was also exploited for fluorescence signal transduction. In addition, click chemistry was used to facilitate receptor synthesis and produce triazole moieties that add to structural rigidity and recognition. In binding studies, both receptors were found to bind $InsP_3$ with high affinity (K_a=0.5–1.0×10^6 M^{-1}) in competitive media (1:1 methanol/water).

1. Introduction

The development of molecular sensors that are effective for detecting important biomolecular targets in competitive media remains a significant challenge in the field of supramolecular chemistry.[1,2] This process involves the design of a macromolecular host containing binding groups that are preorganized to instill geometric complementarity with a target guest and thus increase the affinity and selectivity of binding. In addition, a signal transduction element is installed for detection purposes, of which fluorescence is advantageous due to its high sensitivity.[3–5] Anion sensing is critical as biological systems are highly reliant upon the manipulation of anionic functional groups as a regulatory means, particularly through the control of biomolecule phosphorylation. As a result, sensors for phosphorylated molecules are particularly of interest.[6,7]

Within the field of supramolecular chemistry, anion recognition has proven problematic due to inherent challenges including the large and varying shapes of anions, their multiple pH-dependent charge states, and the difficulty in synthetically incorporating anion-chelating moieties into receptors.[5,8–10] Despite these challenges, a number of receptor motifs have been developed for anion recognition, including polyammonium macrocycles,[11–13] porphyrins,[14] the triethylbenzene system,[15] dimetallic hosts,[16] calixarenes,[17] and steroidal constructs.[18] However, the generation of sensors capable of detecting anionic targets for real world applications remains a significant barrier due to the difficulty of achieving strong binding in competitive media.

An important target for molecular sensing is the signaling molecule inositol (1,4,5)-trisphosphate ($InsP_3$, **2**, Scheme 1). This is the quintessential biologically active inositol phosphate[19–21] due to its prominent role as a second messenger in calcium release, originally reported in 1983.[22] $InsP_3$ is produced through the hydrolysis of phosphatidylinositol (4,5)-bisphosphate by phospholipase C.[23–25] This soluble product then binds to its cognate receptors, the inositol trisphosphate receptors ($InsP_3Rs$),[26–29] which leads to the opening of these gated ion channels, directly resulting in calcium release by gradient diffusion into the cytoplasm. $InsP_3$-mediated calcium release is critical due to the numerous physiological processes that are mediated by this ion, which includes cell proliferation, differentiation, secretion, muscular contraction, embryonic development, immune responses, and many others.[26,29]

Due to the significance of $InsP_3$, this compound has been a target for sensor design. Anslyn and co-workers initially reported a hexaguanidinium receptor with a binding cavity engineered via the 1,3,5-triethylbenzene scaffold.[30] To achieve optical detection, a dye displacement assay was implemented, and strong binding (K_a=2.2×10^4 M^{-1}) was observed in aqueous solution. More recently, Kimura and co-workers took non-fluorescent receptors containing zinc/cyclen chelating groups and incorporated them into a ruthenium tris-pyridyl system to achieve fluorescence signaling.[31] In addition, Ahn and co-workers developed a tris zinc/dipicolylamine receptor, and again employed a dye displacement to achieve detection.[32] Matile and co-workers used a synthetic multifunctional pore based on rigid *p*-octiphenyl rods organized into a β-barrel for fluorimetric detection of $InsP_3$ binding transduced by pore opening and closing.[33] Approaches other than molecular receptors have been successful for $InsP_3$ detection, particularly through the use of labeled or engineered protein receptors.[34–36] Finally, receptors for phytate ($InsP_6$), another inositol phosphate isomer, have been reported as well.[37,38]

While synthetic sensors capable of coordinating $InsP_3$ have been successfully designed, we set out to design a receptor with specific attributes. First, we targeted a system with a built-in fluorescent

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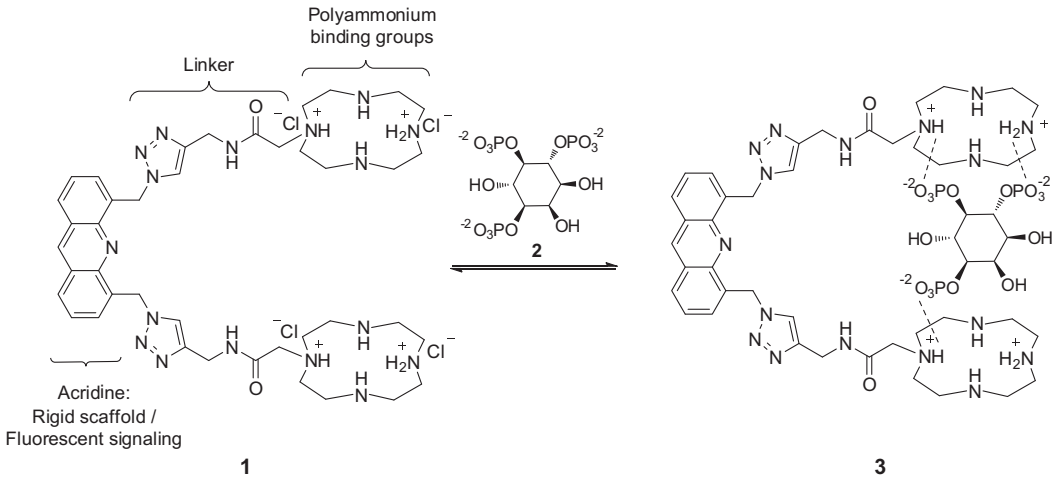


Scheme 1. Design of receptor **1** and hypothetical binding complex with target guest $InsP_3$ (**2**).

signaling group for direct detection of binding.[39] In addition, we sought to implement a rigid scaffold to preorganize binding groups in the creation of a binding cavity. Finally, we looked to exploit the strong affinities of polyammoniums and their zinc complexes for anions[40–44] including phosphate to create a receptor containing two of these groups preorganized into a tweezer structure.

2. Results and discussion

To develop a fluorescent sensor for $InsP_3$, we initially designed host **1** (Scheme 1), which includes a number of features. First, two polyammonium groups were installed to take advantage of the strong anion-binding properties of these moieties. Ditopic polyammonium ligands have previously been investigated, with receptor designs consisting of polyammonium moieties separated by bridging linkers.[11,12,45–57] However, these scaffolds have often been designed around relatively flexible scaffolds, which may be too floppy to maximize preorganization. In order to preorganize these two groups into an effective binding cavity in our receptor, an acridine backbone substituted at the 4- and 5-positions was introduced as a rigid unit for steric gearing. The acridine was also beneficial due to its photophysical properties so as to achieve direct fluorescence signal transduction of guest binding.

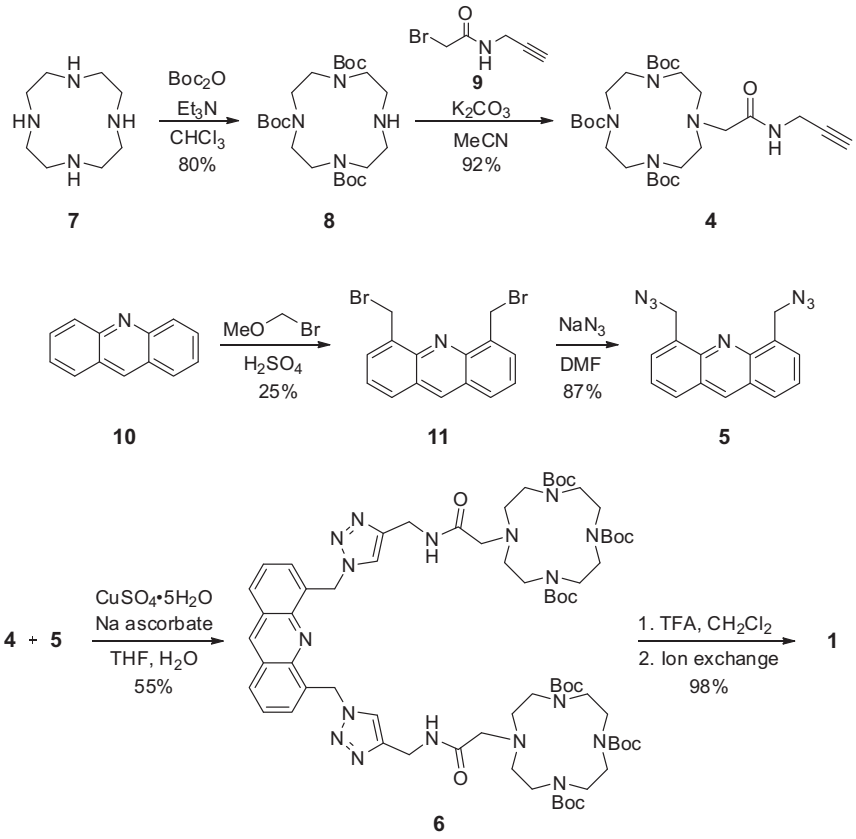
In the host design, triazole-based linkers were incorporated between the acridine scaffold and the polyammonium binding groups, which were also envisaged to be advantageous for multiple reasons. First, this would allow for a convergent receptor synthesis in which these groups could be conveniently merged using click chemistry.[58,59] In addition, the resulting triazole moieties were expected to be beneficial by introducing rigidity to the tether, further assisting in the preorganization of binding groups. Finally, triazoles have also been shown to be effective for forming hydrogen bonding interactions with anionic guests,[60] and thus could contribute to the binding of phosphorylated analytes. In this way, receptor **1** was designed to complement multiply phosphorylated biomolecules, such as $InsP_3$, by forming complexes exemplified by hypothetical model **3** in Scheme 1.

With this receptor design in place, a convergent synthetic route to receptor **1** was devised, as shown in Scheme 2. Here, the key step employed click chemistry to link alkynyl-cyclen intermediate **4** and bis-(azidomethyl)acridine structure **5** to form **6**, which was then deprotected. Ion exchange was next performed to access host **1** with chloride counteranions, and it is estimated that four chlorides are associated with the cationic host structure. In precursor synthesis, cyclen (**7**) was used to access **4** via tris-Boc protection to **8**,[61] followed by coupling with alkyne **9**.[62] Bis-(azidomethyl)acridine **5** was obtained from acridine (**10**) by bis-bromomethylation to **11**,[63] followed by azide substitution. Here, the acridine unit was also beneficial as a result of the convenient derivatization of this precursor at the desired 4- and 5-positions. For binding studies, $InsP_3$ (**2**) was synthesized as described in the Supplementary data.

Following the completion of this synthetic route, receptor **1** was employed for titration binding studies to evaluate the binding of $InsP_3$ (**2**) with concomitant fluorescence signal transduction. First, UV scans of the host were performed, with each exhibiting an absorption peak centered around 355 nm, with excitation at this wavelength leading to an emission signal located around 440 nm. Next, **2** was titrated into host **1** in such a way as to keep the total host concentration constant. Here, dose-dependent decreases in the emission signal of host **1** were observed upon guest addition. This was performed in a range of solvents varying from 0 to 100% methanol in water buffered at pH 7.4. From these studies, a solvent mixture of 50% methanol/water was identified as a competitive solvent in which strong binding was observed. A representative plot indicating the decrease in emission properties of host **1** upon addition of **2** in this solvent mixture is shown in Fig. 1. While the changes in emission properties were somewhat modest, dropping by only 10–15%, these signals could nevertheless be reproducibly correlated to a binding curve for **2**, as shown in Fig. 2. Here, the change in fluorescence intensities upon addition of **2** are shown as averaged values over several runs, with error bars included depicting the standard error. From these results, an association constant of $1.0\times10^6\ M^{-1}$ (Table 1) was calculated for the complex, indicating high-affinity binding in competitive media.

Next, a series of related phosphorylated compounds were employed for binding studies to further investigate the recognition properties of host **1**. These included D-fructose 1,6-bisphosphate trisodium salt (FBP, **12**, Fig. 3), cyanoethyl phosphate barium salt (**13**), and sodium dihydrogen phosphate (**14**), which were selected due to their variation in the number of phosphate and alkyl groups. The binding curves for these guests are shown alongside those corresponding to **2** in Fig. 2, and the resulting association constants are indicated in Table 1. In general, each of these guests exhibited fairly strong binding, indicating that host **1** is generally effective at complexing a range of phosphorylated guest structures in competitive solvents. Nevertheless, slight variations in binding affinity were observed in the order of **2**>**12**>**13**>**14**, indicating very modest selectivity for $InsP_3$, as well as for multiply phosphorylated guests (**2** and **12**) over singly phosphorylated guests (**13** and **14**) in the series.

A benefit of employing polyamine moieties for anion binding is the modular nature of these groups. This is because these receptors



Scheme 2. Synthetic route to bis-cyclen tweezer host **1**.

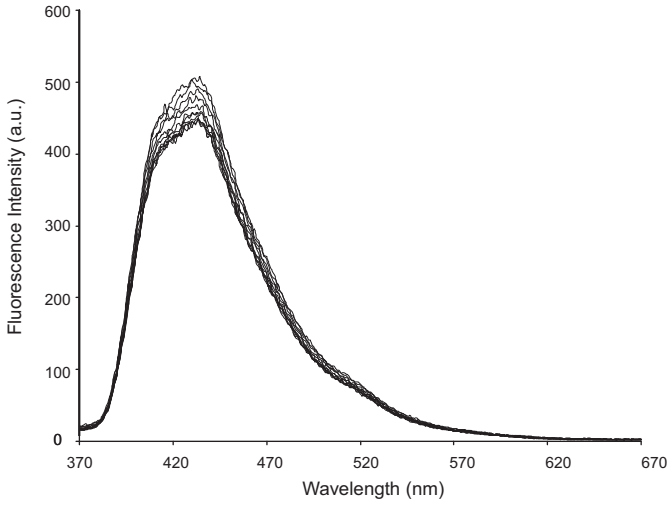


Fig. 1. Plots exhibiting the decrease in the emission properties of host **1** upon addition of guest **2**.

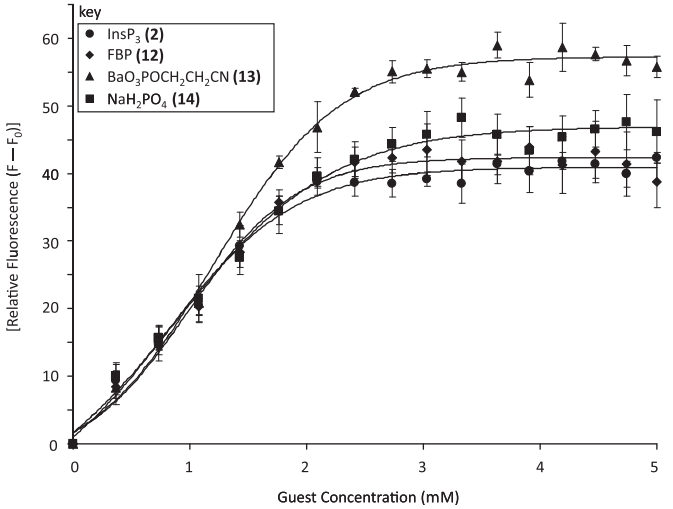


Fig. 2. Binding curves for guests studied in titrations, including inositol 1,4,5-trisphosphate (**2**), fructose 1,6-bisphosphate (**12**), cyanoethyl phosphate (**13**), and sodium dihydrogen phosphate (**14**).

effectively bind anions in both their unchelated polyammonium form, as with receptor **1**, and also when chelated to a metal ion, in which the metal center then acts as the electrophilic binding site. As a result, we next decided to take advantage of this modularity by exploring the binding properties of the bis-zinc complex of this host (**1**-Zn_2, Scheme 3) to compare binding properties. This metalated receptor was synthesized through reaction with zinc acetate.

We next performed titration binding experiments similar to those previously described, this time using host **1**-Zn_2 in order to compare recognition properties. Once again, titration with **2** yielded a decrease in emission for **1**-Zn_2, for which a representative

Table 1
Association constants calculated for the binding of host **1** to phosphorylated guests

Guest	K_a (M^{-1})
Inositol (1,4,5)-triphosphate (**2**)	1.0×10^6
Fructose 1,6-bisphosphate (**12**)	9.5×10^5
Cyanoethyl phosphate (**13**)	8.9×10^5
Sodium dihydrogen phosphate (**14**)	7.8×10^5

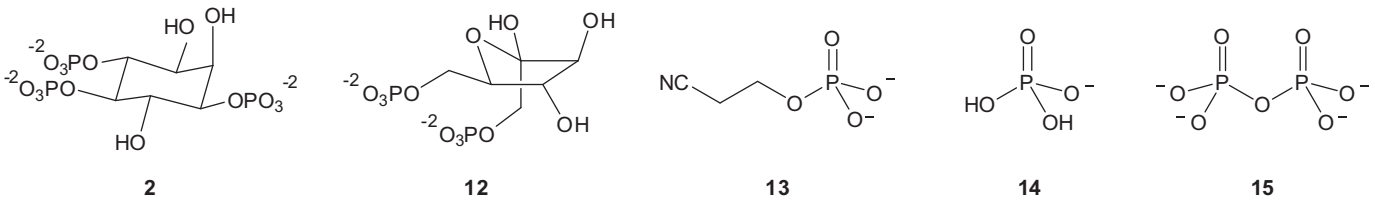
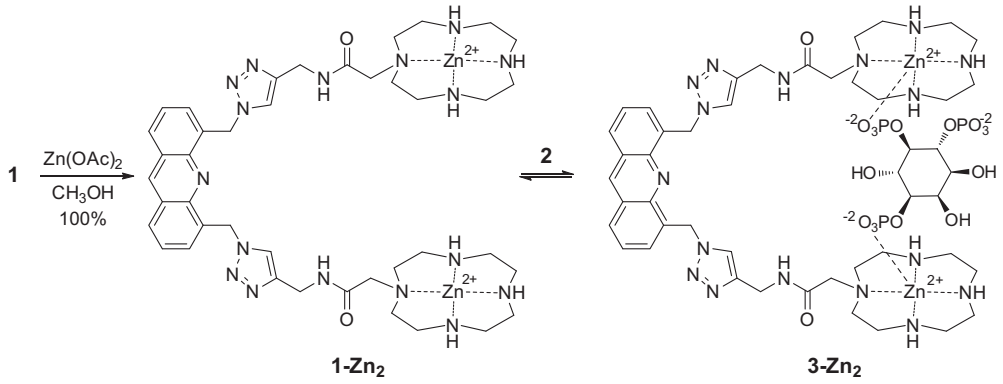


Fig. 3. Structures of guests used for binding studies with receptor **1** and **1**-Zn_2.



Scheme 3. Synthesis of bis-zinc receptor **1**-Zn_2 and hypothetical binding complex with target guest $InsP_3$ (**2**).

plot is shown in Fig. 4. While still modest, the emission changes of **1**-Zn_2 upon addition of **2** (20–25%) were greater than those of receptor **1**. Once again, receptor **1**-Zn_2 exhibited strong binding of **2**, with an association constant of 5.1×10^5 M^{-1} (Table 2). Despite the enhanced fluctuation in emission properties upon guest addition, this binding affinity was slightly weaker than that of receptor **1**. It is possible that host **1** exhibits slightly stronger binding to multiply phosphorylated guests because the positive charge is spread onto multiple ammonium groups, rather than being localized on the two zinc atoms of **1**-Zn_2, which may better complement the charge distribution in these guests.

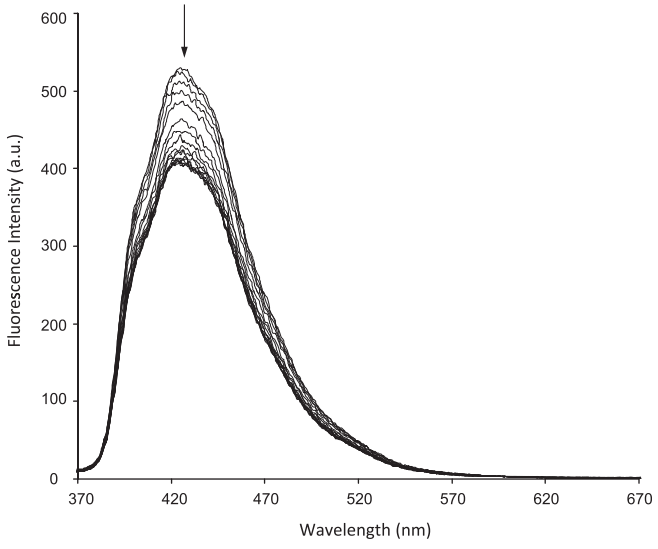


Fig. 4. Plots exhibiting the decrease in the emission properties of host **1**-Zn_2 upon addition of guest **2**.

Next, the same series of phosphorylated guests were analyzed for binding with **1**-Zn_2, along with the addition of sodium pyrophosphate (**15**). The binding curves for these guests are shown alongside those corresponding to **2** in Fig. 5, and the resulting

Table 2
Association constants calculated for the binding of host **1**-Zn_2 to phosphorylated guests

Guest	K_a (M^{-1})
Inositol (1,4,5)-trisphosphate (**2**)	5.1×10^5
Fructose 1,6-bisphosphate (**12**)	4.9×10^5
Cyanoethyl phosphate (**13**)	9.4×10^5
Sodium dihydrogen phosphate (**14**)	4.3×10^5
Sodium pyrophosphate (**15**)	4.7×10^5

association constants are indicated in Table 2. Once again, each guest exhibited strong binding, and only slight variations in affinity were observed. In this case, cyanoethyl phosphate surprisingly yielded the strongest binding, and an ordering of **13**>**2**>**12**>**15**>**14** was observed. However, in these studies multiply phosphorylated guests (**2**, **12**, and **15**) all produced higher changes in fluorescence than those analytes containing a single phosphate group (**13** and **14**). This provided evidence for variation in binding activity

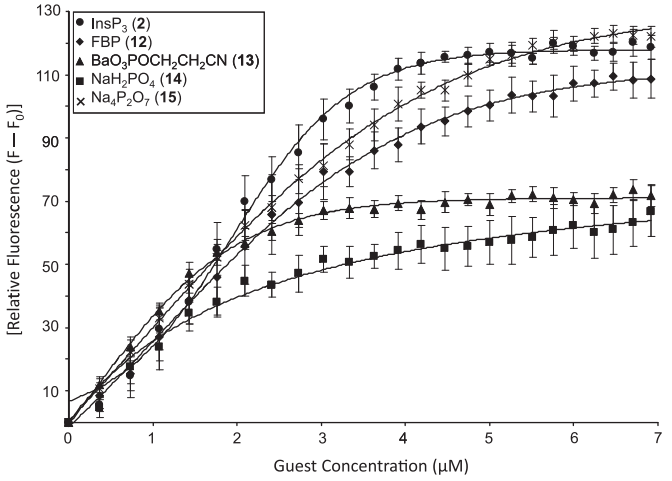


Fig. 5. Binding curves for guests studied in titrations, including inositol 1,4,5-trisphosphate (**2**), fructose 1,6-bisphosphate (**12**), cyanoethyl phosphate (**13**), sodium dihydrogen phosphate (**14**), and sodium pyrophosphate (**15**).

resulting from the presence of more than one phosphate in the guest structure.

3. Conclusions

This article describes the development of fluorescent sensors for $InsP_3$ based upon bis-cyclen tweezer structures either containing free polyammonium or zinc chelated groups. In the design of the host, an acridine backbone was installed as both a fluorescent moiety for signal transduction as well as a rigid scaffold for preorganization. Furthermore, triazole units were implemented for convenient host synthesis via click chemistry, preorganization of cyclen groups due to the rigidity of the triazoles, and since these groups have previously been shown to bind anions. The resulting sensors, **1** and **1**-$\mathbf{Zn_2}$, were both found to bind $InsP_3$ with high affinity in competitive media (methanol/water mix), with detection of binding observed through changes in the host emission spectra upon binding. While receptor **1** exhibited a slightly enhanced binding affinity for $InsP_3$ compared to other phosphorylated guests, this difference was very modest. This is a common obstacle in anion binding as enhancing selectivity remains a significant challenge.

Additionally, receptor **1**-$\mathbf{Zn_2}$ generally benefited from greater emission changes than **1** upon guest addition. Furthermore, the enhanced emission changes of **1**-$\mathbf{Zn_2}$ upon addition of multiply phosphorylated guests as opposed to monophosphate analytes points to the benefits of preorganizing two phosphate binding groups into a cavity for complexation of multiply phosphorylated targets. It is clear that polyammonium macrocycles are highly effective for binding anions, particularly when they are incorporated within preorganized macromolecular structures. However, to fully harness these properties and achieve the ultimate goal of selective recognition, the preorganized scaffold must be carefully designed to maximize interactions with the target guest. To further this goal, we are currently considering alternate templates for enhancing anion-binding specificity.

4. Experimental section

4.1. General experimental

Generally, reagents were purchased from Acros, Aldrich or AK Scientific and used as received. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. Chloride counteranion ion exchange, as well as C18 reverse phase solid phase extraction (SPE) syringe columns were obtained from Silicycle. NMR spectra were obtained using a Varian Mercury 300 spectrometer. Mass spectra were obtained with a JEOL DART–AccuTOF spectrometer and an ABI Voyager DE Pro MALDI spectrometer with high resolution capabilities. UV–vis absorption spectra were obtained with a Thermo Evolution 600 UV–vis spectrophotometer, and fluorescence spectra were obtained with a Perkin Elmer LS 55 luminescence spectrometer. $InsP_3$ (**2**) used for binding studies was synthesized from protected inositol intermediates that we previously reported,[64,65] as described in the Supplementary data.

*4.1.1. 1,4,7-Tris(tert-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (**8**).* A solution of di-*tert*-butyl dicarbonate (1.77 g, 8.12 mmol) in chloroform (10 mL) was added slowly via addition funnel to a solution of cyclen (**7**, 0.51 g, 2.90 mmol) and triethylamine (1.25 mL, 8.99 mmol) in chloroform (40 mL) at rt. The reaction mixture was stirred at rt overnight and then concentrated through rotary evaporation. The resulting residue was purified by column chromatography over silica gel with gradient elution from ethyl acetate to 5% methanol/dichloromethane to yield the product as a white solid (1.02 g, 80%). Characterizations matched previous reports.[61]

*4.1.2. 2-Bromo-N-(propargyl)acetamide (**9**).* Propargylamine (0.36 mL, 5.61 mmol) and triethylamine (0.78 mL, 5.61 mmol) were added to a solution of bromoacetyl bromide (0.50 mL, 5.61 mmol) in dichloromethane (25 mL) at 0 °C. After stirring at 0 °C for 1 h, the reaction mixture was concentrated through rotary evaporation, resuspended in ethyl acetate (50 mL), and then filtered through a pad of silica gel. The filtrate was then concentrated to provide the product as a dark orange solid (0.96 g, 98%). Characterizations matched previous reports.[62]

*4.1.3. N-(Propargyl)-1,4,7-tris(tert-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecan-10-yl-acetamide (**4**).* A solution of **9** (0.19 g, 1.06 mmol) in acetonitrile (5 mL) was added to a solution of **8** (0.34 g, 0.71 mmol) in acetonitrile (20 mL) at rt. Anhydrous potassium carbonate (0.15 g, 1.06 mmol) was then added, and the reaction mixture heated to 80 °C, at which it was stirred for 2 days. The mixture was then filtered, followed by concentration of the filtrate by rotary evaporation. Column chromatography over silica gel with gradient elution from 50 to 100% ethyl acetate/hexanes then provided the product as a white solid (0.37 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.16 (s, 1H), 4.05–3.98 (m, 2H), 3.71–3.11 (m, 14H), 2.92–2.45 (m, 4H), 2.18 (s, 1H), 1.51–1.43 (m, 27H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.72, 155.52, 79.88, 79.18, 71.18, 58.19, 56.22, 50.95, 49.78, 28.50. DART–HRMS $[M+H]^+$ calcd: 568.3710, found: 568.3605.

*4.1.4. 4,5-Bis(azidomethyl)acridine (**5**).* Sodium azide (0.51 g, 7.82 mmol) was added to a solution of 4,5-bis(bromomethyl)acridine[63] (0.48 g, 1.30 mmol) in *N*,*N*-dimethylformamide (20 mL) at rt. The reaction mixture was then heated to 80 °C, and allowed to stir overnight. The reaction crude was then filtered, and the filtrate was concentrated by rotary evaporation. Column chromatography over silica gel with gradient elution from 20 to 100% dichloromethane/hexanes gave the product as a light yellow solid (0.33 g, 87%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.72 (s, 1H), 7.94 (d, J=8.5 Hz, 2H), 7.78 (d, J=6.8 Hz, 2H), 7.52 (dd, J=8.5, 6.8 Hz, 2H), 5.20 (s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.43, 136.59, 134.41, 129.77, 128.61, 126.60, 125.67, 51.49. DART–HRMS $[M+H]^+$ calcd: 290.1154, found: 290.1074.

*4.1.5. Hexa-tert-butyl 10,10′-((((1,1′-(acridine-4,5-diylbis(methylene))bis(1H-1,2,3-triazole-4,1-diyl))bis(methylene))bis(azanediyl))bis(2-oxoethane-2,1-diyl))bis(1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate) (**6**).* A solution of alkyne **4** (0.084 g, 0.15 mmol) in tetrahydrofuran (1 mL) was added to a solution of bis-azide **12** (0.021 g, 0.074 mmol) in tetrahydrofuran (1 mL) at rt. Copper sulfate pentahydrate (0.0092 g, 0.037 mmol), sodium ascorbate (0.037 g, 0.19 mmol), and water (2 mL) were then added, and the reaction mixture was stirred at rt overnight. After the addition of water (20 mL), the mixture was extracted with dichloromethane (2×20 mL). The combined organic layers were then dried over magnesium sulfate, filtered, and concentrated. Column chromatography over silica gel with gradient elution from 3 to 10% methanol/dichloromethane provided the product as a pale yellow solid (0.058 g, 55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.03 (d, J=8.5 Hz, 2H), 7.82 (s, 2H), 7.71 (d, J=6.2 Hz, 2H), 7.58–7.49 (m, 2H), 7.37 (s, 2H), 6.30 (s, 4H), 4.45 (d, J=5.0 Hz, 4H), 3.52–3.00 (m, 28H), 2.62 (s, 8H), 1.52–1.30 (m, 54H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.98, 167.27, 146.06, 137.11, 129.30, 126.60, 125.78, 79.80, 50.45, 47.88, 34.97, 28.43. MALDI–HRMS $[M+Na]^+$ calcd: 1446.8238, found: 1446.8245.

*4.1.6. N,N′-((1,1′-(Acridine-4,5-diylbis(methylene))bis(1H-1,2,3-triazole-4,1-diyl))bis(methylene))bis(2-(1,4,7,10-tetraazacyclododecan-1-yl)acetamide) (**1**).* Trifluoroacetic acid (3 mL) was added dropwise to a solution of **6** (0.024 g, 0.017 mmol) in dichloromethane (3 mL) at rt. The reaction mixture was stirred at rt for 3 h, followed by concentration via rotary evaporation. The crude product was next dissolved in deionized water, loaded onto

a chloride counteranion exchange column (0.5 g, Si-TMA chloride), and eluted with deionized water. Purification on a reversed-phase column (2 g, C18) with gradient elution from 0 to 50% methanol/water gave the product as a yellow solid (0.016 g, 98%). ^{1}H NMR (300 MHz, CD_3OD) δ 9.09 (s, 1H), 8.19 (d, J=8.3 Hz, 2H), 8.04 (s, 2H), 7.78 (d, J=6.6 Hz, 2H), 7.67–7.59 (m, 2H), 6.36 (s, 4H), 4.50 (s, 4H), 3.39 (s, 4H), 3.24–2.70 (m, 32H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.06, 147.03, 145.90, 138.48, 134.30, 132.21, 130.69, 127.88, 126.84, 124.95, 56.87, 51.73, 51.43, 47.85, 45.93, 44.25, 44.01, 35.76. MALDI–HRMS $[M+HCl+H_2O+H]^+$ calcd: 878.5145, found: 878.5143.

*4.1.7. Zinc(II) complex (**1**-**Zn**$_2$).* Zinc acetate (0.008 g, 0.044 mmol) was added to a solution of **1** (0.021 g, 0.022 mmol) in methanol (5 mL). The reaction mixture was stirred at 50 °C overnight, followed by concentration via rotary evaporation to obtain the tetraacetate complex as a light yellow solid (0.026 g, quant.). ^{1}H NMR (300 MHz, CD_3OD) δ 9.11 (s, 1H), 8.21 (d, J=8.8 Hz, 2H), 8.06–7.90 (m, 2H), 7.90–7.76 (m, 2H), 7.69–7.60 (m, 2H), 6.34 (s, 4H), 4.49 (s, 4H), 3.45 (s, 4H), 3.04–2.62 (m, 32H). MALDI–HRMS $[M-3H]^+$ calcd: 952.3505, found: 952.3497.

4.2. Procedure for titration binding studies

All binding studies were conducted in a buffered solution of 50% methanol/water solution (50 mM Tris buffer, pH 7.4) formed by adding a 2× buffered aqueous solution to methanol. After obtaining an excitation spectrum of host **1**-**Zn**$_2$ fluorescence experiments were performed through excitation at 355 nm at rt. In these studies, a 10 μM buffered solution of host **1**-**Zn**$_2$ (2 mL) was titrated stepwise with 25 μL aliquots of a buffered solution consisting of 10 μM host **1**-**Zn**$_2$ and 30 μM guest. After each addition, the fluorescence spectra were recorded. The changes in fluorescence intensities at the emission λ_{max} (440 nm) were then plotted against the concentration of added guest. Binding constants were next determined in two ways. First, the data were analyzed by non-linear regression analysis using saturation curve fitting in SigmaPlot.[66] Apparent K_d values were also evaluated using the half-maximal point of the binding curves, and both methods resulted in similar values. It should be noted that binding curves exhibit some sigmoidal, or s-shaped, character, and thus are depicted using a four-parameter sigmoidal curve fit in Fig. 2. Sigmoidal properties typically indicate the presence of more than one equilibria or cooperativity.

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Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2011.03.092.

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