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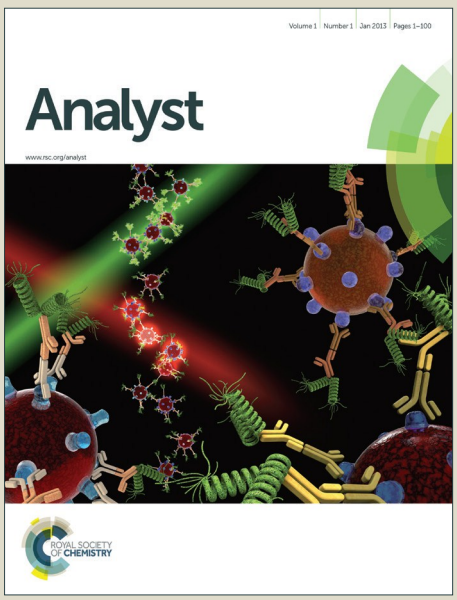

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A tool for the selective sequestration of ATP and PP_i to aid in-solution phosphopeptide detection assays

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The presence of small phospho-anions, such as PP_i and ATP in protein samples often complicates the robust detection of phosphoproteins by metal-based chemosensors and receptors. We herein report the development of a bis(Zn^{2+}-cyclen)-triethylbenzene scaffold which can selectively sequester PP_i and ATP without affecting the detection of a di-phosphorylated peptide by a ProxyPhos chemosensor.

The selective detection of phosphorylated proteins is an important area of research, and several efficient synthetic receptors have been developed for this purpose. Some prominent examples include ProQ Diamond [1] and Phostag [2] for the detection of phosphoproteins on polyacrylamide gels and blotting membranes.[1,3-8] The majority of synthetic receptors which are capable of phosphoprotein detection incorporate a Lewis-acid metal centre(s) which recognizes and binds to the anionic phosphate. However, this metal-based detection mode often suffers from off-target effects arising from binding to small phospho-anions such as PP_i and ATP,[9] which are often abundant in protein samples. To avoid this off-target effect, size-exclusion chromatography,[10] protein immobilization, and precipitation steps[11] can be used prior to the application of these phosphoprotein detection agents. However, this introduces extra steps in the phosphoprotein detection protocol and limits the application of these detection tools in aqueous solutions. Thus, simple methods for the selective sequestration of phospho-anions could be of value to the phosphoprotein detection field.

Inspired by previously reported applications of the pre-organized triethylbenzene (TEB) scaffold for the selective binding of low-molecular weight (MW), phospho-anions,[12] we aimed to develop an analogous scaffold which would selectively sequester phospho-anions, over both phosphorylated peptides and protein sites. To achieve this, we synthesized receptor **1** (Figure 1), which was designed to include two Zn^{2+}-cyclen phosphate recognition units, tethered to the TEB scaffold via indole linkers. We hypothesized that the resultant cavity formed by the indole linkers would sterically prevent receptor **1**'s association with phosphopeptides but not with more compact phosphoanions. In order to determine the selectivity of receptor **1**, we assessed its binding potency to low-MW phospho-anions (sodium salts of adenosine triphosphate (ATP), adenosine monophosphate (AMP), pyrophosphate (PP_i), sulphate (SO_4^{2-}) and phenyl phosphate (PhoP)) as compared to phosphopeptides (Ac-ApYpYAA-NH_2 and Ac-AYpYAA-NH_2). To determine the relative binding potencies of receptor **1** to the aforementioned targets, an established pyrocatechol violet (PV)-based indicator displacement assay (IDA) was employed.[13,14]

Upon the association of PV with the Zn^{2+}-cyclen units of receptor **1**, the absorbance maximum of PV is found at 650 nm

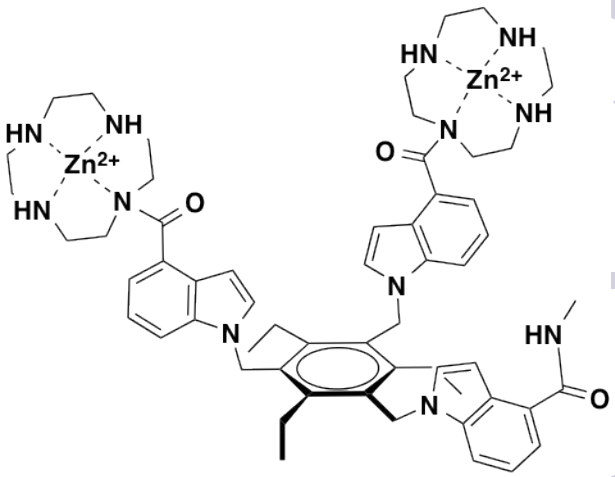

Figure 1. Structure of receptor **1**

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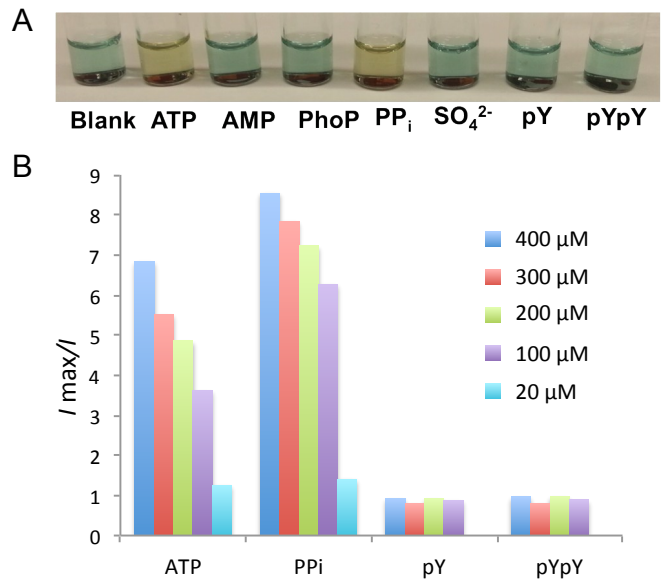

Figure 2. Displacement of PV from receptor **1** in response to low-MW anions and phosphopeptides. (A) Image of [receptor **1**: PV] complex (20 μM each) after the addition of 100 μM of analyte; (B) Titration of [receptor **1**:PV] complex (20 μM each) with various concentrations of analytes. Absorbance was measured at 650 nm using BioTek Cytation 3. I max is the maximum absorbance of [receptor **1**:PV] complex in the absence of analytes; I is the observed absorbance of [receptor **1**:PV] complex in the presence of an analyte. All experiments were performed in 50 mM HEPES, 5% DMSO, pH 7.2.

and the solution appears blue to the naked eye (Figure S1). In the presence of an analyte, which competes for binding to the Zn^{2+}-cyclen units of receptor **1**, the PV is displaced, and its absorbance maximum shifts to 450 nm resulting in a change in colour of the solution, going from blue to yellow. Thus, to determine the relative binding potencies, we pre-incubated 20 μM of receptor **1** with 20 μM of PV, and of various analytes (100 μM) were added to this receptor **1**:PV complex in 50 mM HEPES, 5% DMSO, pH 7.2. As can be seen from Figure 2A, only ATP and PP_i induced a visible colour change, indicating that receptor **1** bound ATP and PP_i in preference to the phosphopeptides, as was originally hypothesized. Furthermore, no binding of receptor **1** to phosphopeptides was observed at concentrations of up to 400 μM of phosphopeptide (Figure 2B). Therefore, selective binding of ATP and PP_i over phosphopeptides by receptor **1** can be achieved over a range of analyte concentrations.

To probe whether receptor **1** could selectively sequester PP_i in an established phosphopeptide detection method, we applied it to the in-solution ProxyPhos assay.[15] ProxyPhos was designed to sense proximally phosphorylated peptide and protein motifs (Figure 3A).[15] However, as with many metal-based sensors, ProxyPhos has been shown to detect pyrophosphate (Figure 3A). We reasoned that, since receptor **1**

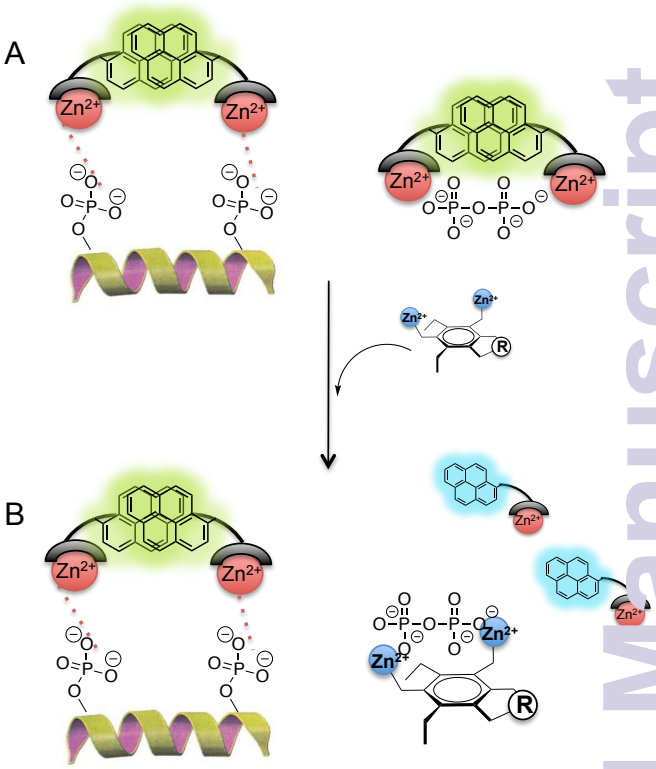

Figure 3. Proposed scheme of the selective sequestration of PP_i by receptor **1** in a ProxyPhos phosphopeptide detection assay.

preferentially binds PP_i over phosphopeptides, the addition of receptor **1** to a ProxyPhos assay solution would reduce the off-target signal from PP_i without affecting phosphopeptide detection (hypothesis illustrated in Figure 3B).

To probe this hypothesis, we first incubated ProxyPhos (40 μM) with its target di-phosphorylated pYpY peptide (10 μM), PP_i (10 μM) or a mixture of both (10 μM each; Figure 4) in 50 mM HEPES, 5% DMSO, pH 7.2. The ProxyPhos fluorescence signal was measured at 476 nm (20 nm bandwidth) upon excitation at 350 nm. As can be seen in Figure 4 (red bars), both the di-phosphorylated peptide and PP_i induced ProxyPhos signal, with higher signal in response to PP_i (Figure 4 red bars). Thus, when the detection of a phosphopeptide is of interest and PP_i impurities are present, the concentration of a phosphopeptide is over-estimated in the presence of PP_i (Figure 4, red bar "pYpY+PP_i"). The addition of receptor **1** (40 μM) to this ProxyPhos assay does not interfere with the ProxyPhos chemosensor signal itself (Figure 4, blue bar "no analyte"). However, the presence of receptor **1** (40 μM) significantly reduces ProxyPhos signal in response to PP_i (75% signal reduction), yet does not greatly affect ProxyPhos signal in response to the phosphopeptide (29% signal reduction; Figure 4 blue bars). The presence of receptor **1** (40 μM) in the phosphopeptide-PP_i mixture restored the ProxyPhos signal to the original level as found in the solution of the

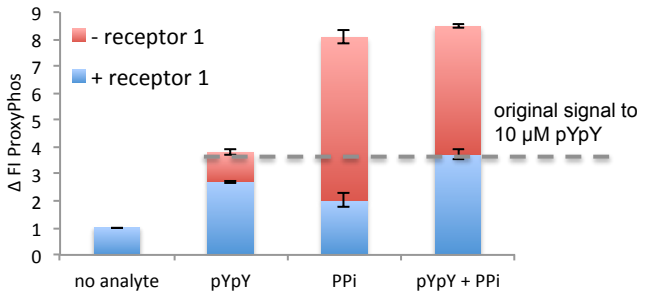

Figure 4. ProxyPhos (40 μM) signal in the presence of pYpY (10μM), PP_i (10μM) and pYpY+PP_i (10 μM each) with (blue bars) and without (red bars) 40μM receptor **1**. The fluorescence emission of ProxyPhos was measured using TECAN Infinite M1000 using 350 nm excitation (5 nm bandwidth) and 476 nm emission (20 nm bandwidth). ΔFI represents the ratio of ProxyPhos signal in the presence and in the absence of analyte. Dashed line marks the level of ProxyPhos signal to pYpY when no off-target components (*e.g.* PP_i) are present.

phosphopeptide without PP_i (Figure 4, blue bar, dashed line). Collectively, these result support our proposed hypothesis that receptor **1** is able to sequester PP_i in aqueous solutions without significantly affecting phosphopeptide detection.

Conclusions

In conclusion, we have demonstrated that the rigid bis(Zn^{2+}-cyclen)-TEB scaffold can selectively associate with small phospho-anions, including PP_i and ATP, in preference to proximally- and mono-phosphorylated sites found on peptides. This feature of receptor **1** can be effectively exploited in phosphopeptide, and possibly, protein detection assays to sequester small-molecule phosphoanions that can trigger off-target signaling. In addition, the synthetic route developed for this scaffold allows for the facile functionalization of this receptor at the arm which is not occupied by a Zn^{2+}-cyclen. This opens possibilities for the immobilization of receptor **1** or further tuning of its selectivity and affinity towards small biologically relevant phosphoanions.

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‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.
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