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Design and Synthesis of Supramolecular Phosphatases Formed from a Bis(Zn^{2+}-Cyclen) Complex, Barbital-Crown-K^+ Conjugate and Cu^{2+} for the Catalytic Hydrolysis of Phosphate Monoester

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The development of artificial mimics of natural enzymes such as hydrolases and phosphatases is one of the great challenges in bioorganic and bioinorganic chemistry and related sciences. Supramolecular strategies are one of the useful methods to construct artificial catalysts as mimics of natural enzymes and to understand their reaction mechanisms. Herein, we report on the formation of amphiphilic supramolecular phosphatases by the 2:2:2 self-assembly of a bis(Zn^{2+}-cyclen) complex (cyclen = 1,4,7,10-teraazacyclododecane) containing a 2,2′-bipyridyl (bpy) linker and one long alkyl chain (Zn_2L^3), 5,5-diethylbarbituric acid (Bar) derivative functionalized with 1-aza-18-crown-6 ether and Cu^{2+} in a two-phase solvent system ($CHCl_3/H_2O$). We hypothesized that crown ether moiety of the Bar-crown ether conjugate would form complexes with alkaline ions and other metal ions such as Li^+, Na^+, K^+, Rb^+, Mg^{2+} and La^{3+} in organic phase to mimic the Mg^{2+} found as the third metal ion in the active site of alkaline phosphatase (AP). The results indicate that the 2:2:2:4 complexes of Zn_2L^3, a Bar block equipped with the 18-crown-6 ether, Cu^{2+} and alkaline metal are constructed in a two-phase solvent system. The resulting complexes have a higher hydrolysis activity for mono(4-nitrophenyl)phosphate (MNP) in the presence of K^+ than that in the presence of Li^+, Na^+, Rb^+, Mg^{2+} and La^{3+} and a greater hydrolysis activity than our previous supermolecules having no crown ether part, suggesting that crown ether-K^+ complex located in close proximity to the $Cu_2(\mu\text{-}OH)_2$ core contributes to the acceleration of the MNP hydrolysis.

Introduction

The phosphorylation and dephosphorylation of proteins and related biomolecules are essential regulatory reactions associated with signal transduction and numerous cellular functions. The structure, subcellular localization and stability of such molecules can have a significant impact on the biological activity of such proteins. Phosphorylation is accomplished via a kinase action, as exemplified by tyrosine kinases which are related to signal transduction in living cells.[1] Indeed, several molecular targeted drugs that target tyrosine kinases in cancer cells and inhibit their action have been developed for the treatment of cancer.[2] At the same time, dephosphorylation via phosphatases is a vital switching-on and -off mechanism in cell signaling. Dephosphorylation is promoted by protein phosphatases such as alkaline phosphatase (AP), which contains two zinc ions (Zn^{2+}) and one magnesium (Mg^{2+}) ion in its active center, as revealed by the X-ray crystal structure analysis of AP.[3] These ions function not only to confer catalytic activity for the hydrolysis of monoesters of phosphoric acid but also for transphosphorylation reactions that proceed in the presence of high concentrations of phosphorylation acceptors.[4]

A number of artificial phosphatases that mimic the active center of metallophosphatases have been reported to date.[5] However, only a very few of them function as catalysts for the hydrolysis of a phosphate monoester such as mono(4-nitrophenyl) phosphate (MNP), a typical model substrate of phosphate hydrolysis. Hence, dephosphorylation by artificial catalysts that mimic protein phosphatases remains a great challenge because phosphate monoesters are generally less reactive than phosphate diesters and triesters.[6] The synthesis of artificial enzyme models that are constructed by covalent bonds typically requires long and tedious synthetic routes especially for their functionalization, which is one of the drawbacks of such artificial systems.[7]

A supramolecular strategy that utilizes the self-assembly of artificial molecular building blocks equipped with appropriate functionalities could be a powerful approach to overcome the aforementioned drawbacks.[8–10] In this context, we previously reported on the formation of the supramolecular complex **8a**, by the 2:2:2 assembly of a bis(Zn^{2+}-cyclen) complex (cyclen = 1,4,7,10-tetraazacyclododecane) containing the 2,2'-bipyridyl (bpy) linker **1** (Zn_2L^1), a dianion of barbital **4a** (Bar), and a copper(II) ion in an aqueous solution (Scheme 1a).[11] The complex is stabilized mainly by coordination bonds between imide anions of the Bar units and Zn^{2+} ions and hydrogen bonds between the NH protons of Bar units and the NH groups

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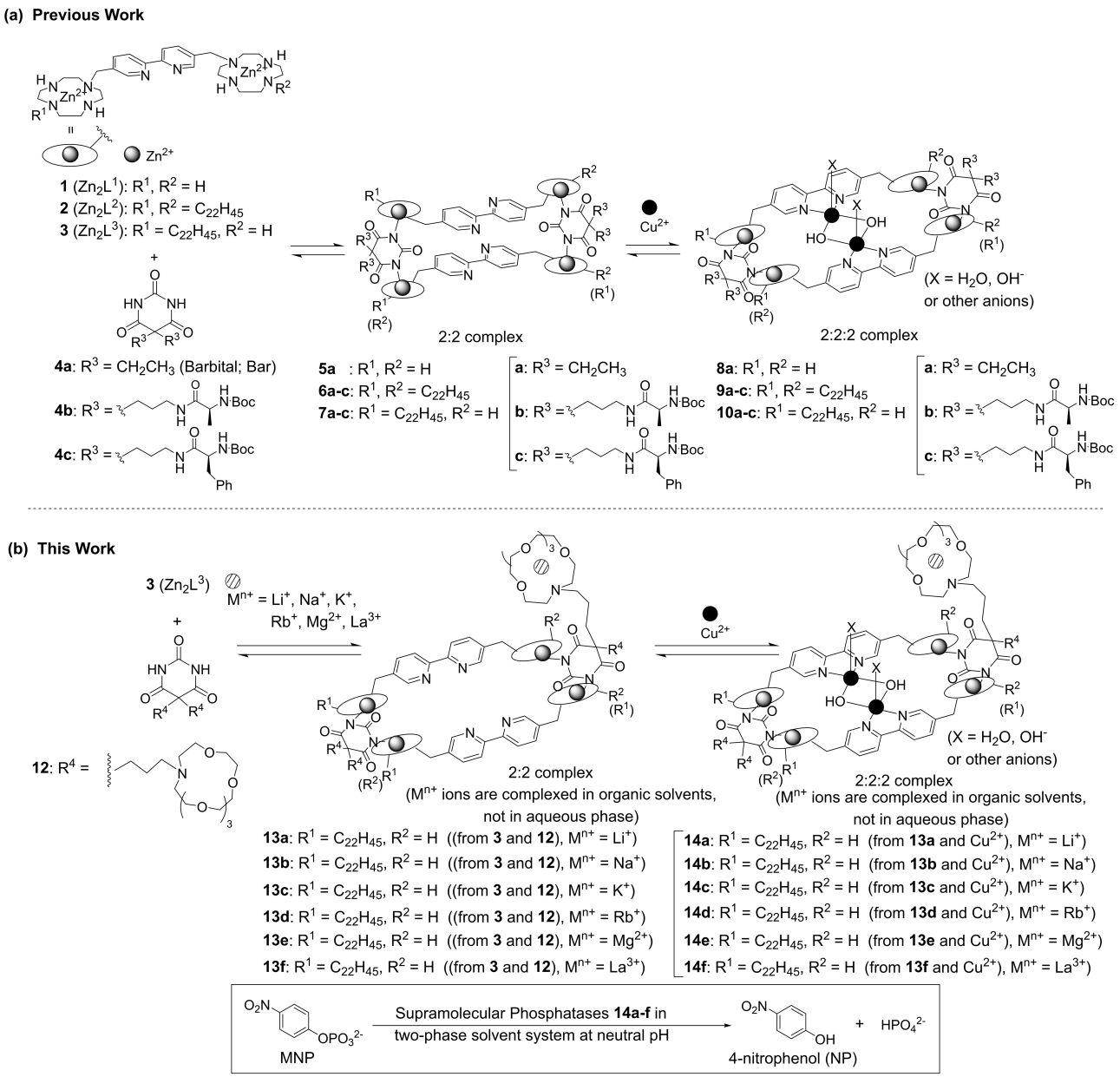
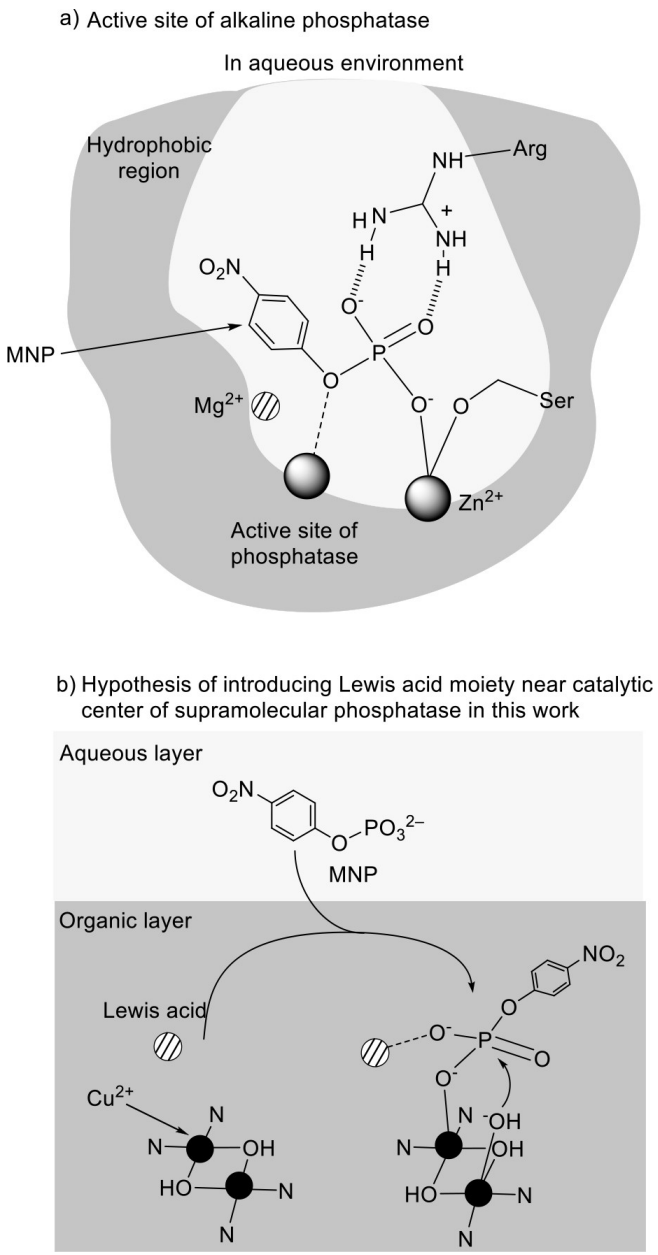


Scheme 1. (a) Formation of the 2:2:2 supramolecular complexes **8**–**10** by the self-assembly of Zn_2L complexes (**1**–**3**), 5,5-diethylbarbital (Bar) derivatives (**4a**–**c**) and Cu^{2+} in an aqueous solution at neutral pH or in a two-phase solvent system ($CHCl_3/H_2O$) that was used in our previous study. (b) Proposed scheme for the formation of the 2:2:2 supramolecular complexes **14a**–**f** by the self-assembly of **3** (Zn_2L^3), a Bar derivative having 1-aza-18-crown-6 ether (**12**) and Cu^{2+} in the presence of other metals (e.g. Li^+, Na^+, K^+, Rb^+, Mg^{2+} and La^{3+}) in a two-phase solvent system ($CHCl_3/H_2O$).

of cyclen, as disclosed by an X-ray crystal structure analysis. It was discovered that **8a** contains a $Cu_2(\mu\text{-}OH)_2$ core, which resembles the active centers of metallophosphatases such as AP, and that one of the ethyl groups of the Bar unit is located in close proximity to the $Cu_2(\mu\text{-}OH)_2$ core. More importantly, **8a** was found to accelerate the hydrolysis of MNP, although the yield was low, possibly due to product inhibition by inorganic phosphate (HPO_4^{2-}), a byproduct of the reaction.

Scheme 2a is a schematic presentation of the hydrophobic active site of AP, in which the substrate (MNP) is activated by the two Zn^{2+} ions and then undergoes hydrolysis. To mimic this situation, we constructed the hydrophobic supramolecular complexes **9a**–**c** and the amphiphilic supramolecular complexes **10a**–**c** (Scheme 1a) by the 2:2:2 assembly of a bis(Zn^{2+}-cyclen) complex containing two long alkyl chains **2** (Zn_2L^2)[12] and a complex that contained one long alkyl chain **3** (Zn_2L^3),[13] respectively, with the functionalized Bar derivatives and Cu^{2+} in a two-phase solvent system ($CHCl_3/H_2O$). It was expected that the hydrophobic or amphiphilic 2:2:2 complexes **9** or **10** would be formed mainly in the organic layer, and that product inhibition by HPO_4^{2-} could be reduced, since the hydrophilic HPO_4^{2-} would be released into the aqueous layer with the $Cu_2(\mu\text{-}OH)_2$ core being regenerated. Indeed, **9** and **10** were both found to accelerate the hydrolysis of MNP and their catalytic



Scheme 2. (a) Schematic presentation of the active center of AP containing two Zn^{2+} ions and one Mg^{2+} ion. (b) Hypothesis for mimicking the active center of AP, where two Cu^{2+} ions are present in the active site and other metals as Lewis acidic sites would be introduced into the close proximity to the $Cu_2(\mu\text{-}OH)_2$ site, in the organic layer of the two-phase solvent system.

turnover numbers (CTNs) were 2~4 (CTN = 2~2.7 for **9b–c** and 3~4 for **10a**).[12,13] One of most important findings was that the hydrolysis of MNP by **9** and **10** in the two-phase solvent system obeyed Michaelis-Menten kinetics, suggesting that these reaction systems consisting of a supramolecular complex and two-phase solvent system mimic the active sites of AP reasonably well.

It is also known that the active site of AP contains Mg^{2+}, as the third Lewis acidic ion, which generates Mg^{2+}-bound OH^- ion that activate Ser (ser102) residue.[3,4i] In this work, we decided to add the third Lewis acid to the $Cu_2(\mu\text{-}OH)_2$ active sites of our previous supramolecular phosphatases, as shown in Scheme 2b.

It is well known that crown ethers and their derivatives are capable of forming complexes with hard metal cations such as alkaline metals, alkaline earth metals and lanthanides.[14–16] Therefore, the side chains of the Bar units were functionalized with 1-aza-18-crown-6 ether in this work (**12** in Scheme 1b), because one of the two ethyl groups of each Bar unit in **8** is located in close proximity to the $Cu_2(\mu\text{-}OH)_2$ catalytic site,[11] as described above. Since it is also known that the complexes of crown ethers with alkali metal ions are more stable in organic solvents than in an aqueous phase, we hypothesized that alkaline and alkaline earth metal ions such as Li^+, Na^+, K^+, Rb^+ and Mg^{2+}[16f–h] and lanthanide ions[16i–k] contained in the aqueous buffer solution would be extracted into the organic layer by the complexation with the crown ether moiety of the Bar unit in the supramolecular complexes (Scheme 3). We expected that these alkaline (and alkaline earth) metals would assist in the activation of MNP, resulting in a more efficient hydrolysis, as predicted in Scheme 2b. In this paper, we report on the construction of 2:2:2 supramolecular complexes (**14a–f** in Scheme 1b and Scheme 3) from **3** (Zn_2L^3) with Bar derivative equipped with 1-aza-18-crown-6 ether (**12**) and Cu^{2+} in the presence of additional metals (e.g. Li^+, Na^+, K^+, Rb^+, Mg^{2+} and La^{3+}). To the best of our knowledge, model systems containing such a complicated and sophisticated architecture have not been previously reported.

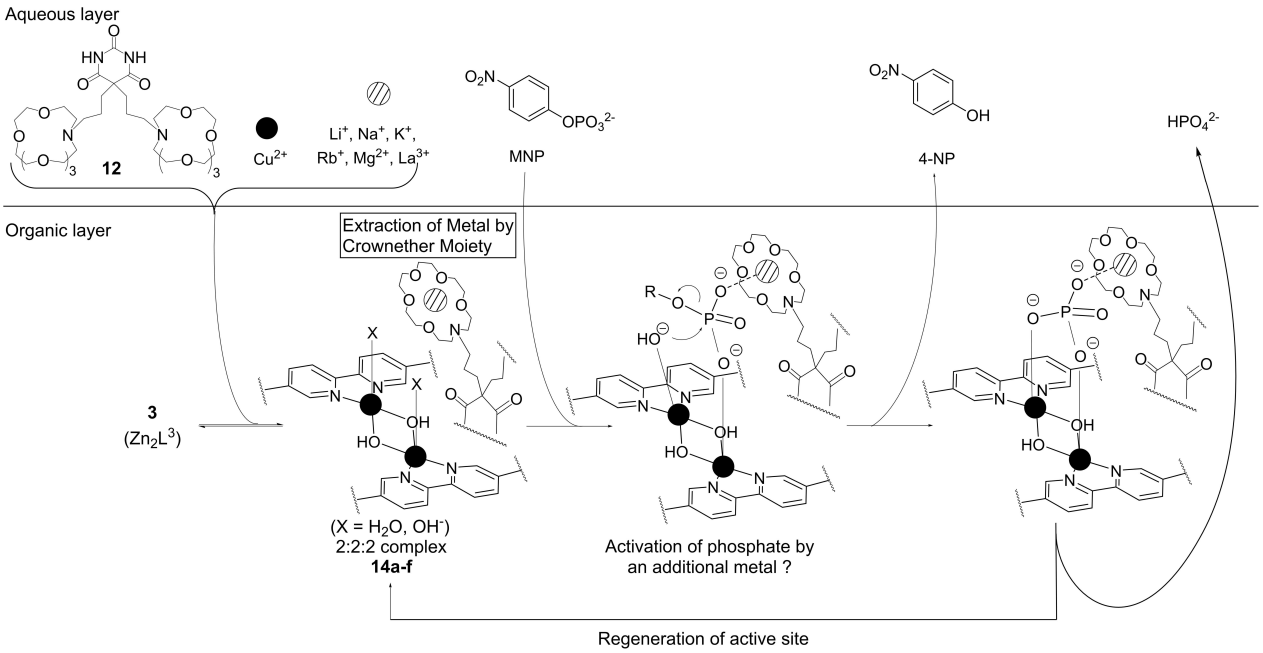
Results and Discussion

Proposed Structure of 14c

Prior to the synthesis of **12**, the three-dimensional structure of **14c** (formed from **3**, **12**, Cu^{2+} and K^+) was speculated using "Biovia Discovery 2020 client" software based on the crystal structure of **8a**[11] (Figure 1a, in which Cu^{2+}-bound H_2O molecules are omitted for clarity). As shown in Figure 1b, in which two C_{22} alkyl groups are assumed to be oriented in the same direction (so called *cis* structure), it was expected that two of the four azacrown ether-K^+ complex units of **14c** would be located close to its $Cu_2(\mu\text{-}OH)_2$ site by choosing a C_3 linker between the Bar and the azacrown units.

Synthesis of the Barbital-18-Crown-6 Conjugate 12

The barbital derivative functionalized with 1-aza-18-crown-6 ether **12** was synthesized as shown in Scheme 4. Compound **18** was synthesized from diethyl malonate **11** and **15** via **16** and **17**, according to our previous papers[12,13] and was reacted with 1-aza-18-crown-6 ether to afford **12**.



Scheme 3. Proposed scheme for the hydrolysis of MNP by amphiphilic supramolecular complexes (**14a**–**f**) having a crown ether-alkaline (alkaline earth) metal complex formed from **3** (Zn_2L^3), **12**, Cu^{2+} and additional metal ions for the hydrolysis of MNP in a two-phase solvent system.

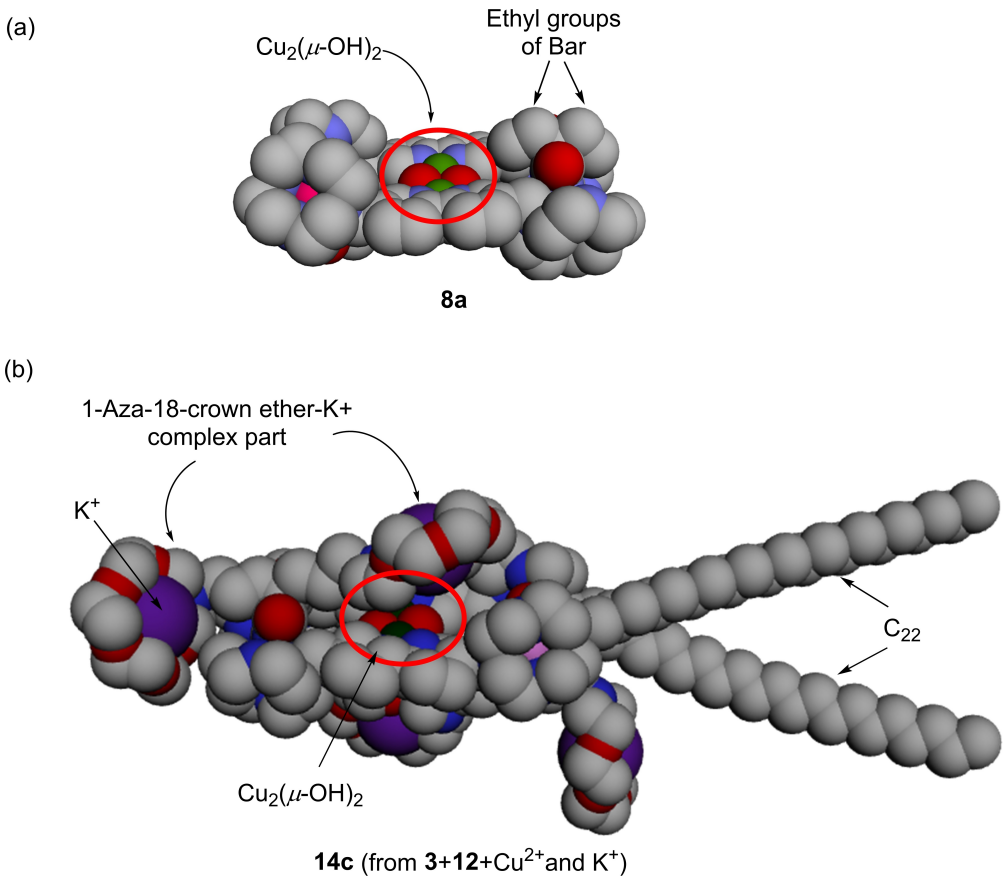
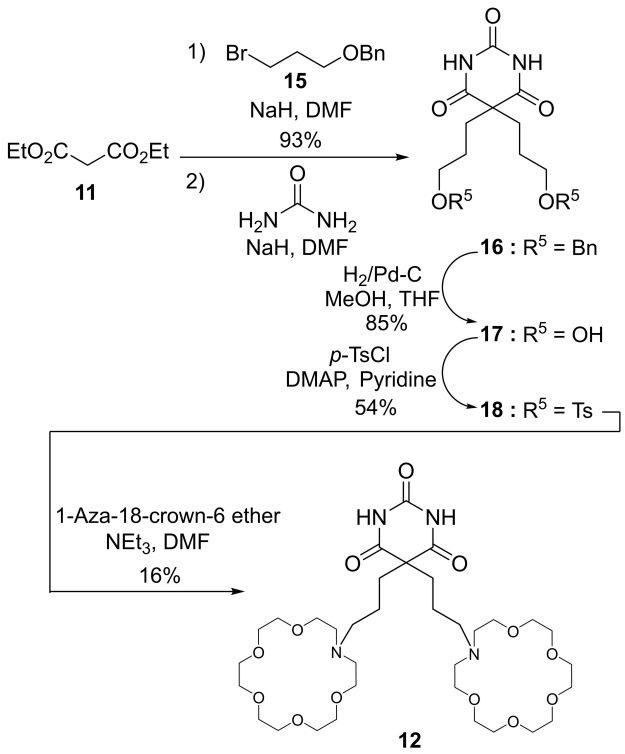


Figure 1. (a) X-ray crystal structure of **8a**[11] and (b) proposed structure of **14c** (assuming a *cis* form, in which two alkyl groups are orientated to the same side).



Scheme 4. Synthesis of the barbital-18-crown-6 conjugate **12** as a building unit for supramolecular complexes **14a–f**.

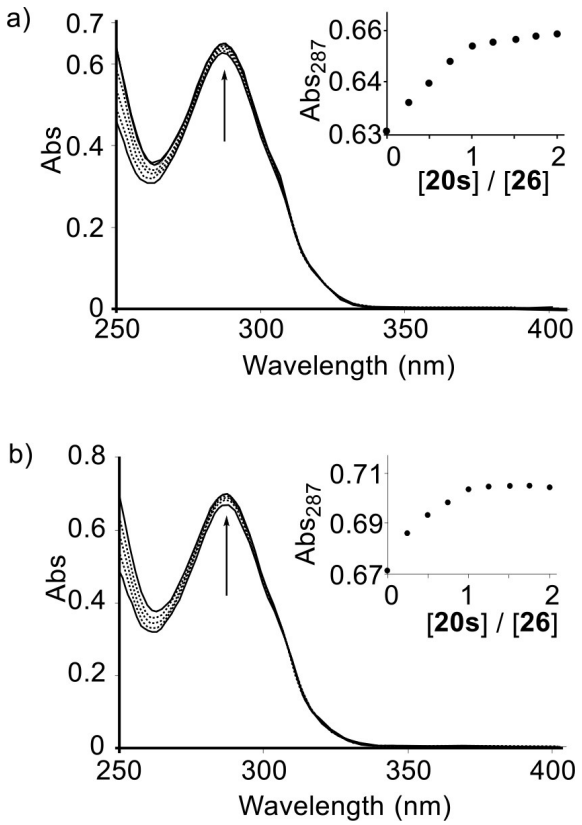


Figure 2. (a) UV/Vis titrations of **3** (40 μM) with **12** in the presence of an excess amount of Na^+ (0.05 M) in DMSO/50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$)) (4/96) at 37 °C. (b) UV/Vis titration of **3** (40 μM) upon the addition of **12** in the presence of an excess amount of K^+ (0.05 M) in DMSO/50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3)) (4/96) at 37 °C.

Complexation Behavior of 3 (Zn_2L^3) with 12 and Cu^{2+} as Studied by UV/Vis Titration in the Presence of an Excess Amount of Na^+ or K^+

The formation of the 2:2 supramolecular complex **13** from the bis(Zn^{2+}-cyclen) complex **3** (Zn_2L^3) and **12** was predicted in Scheme 1b and checked by the UV/Vis titration of **3** with **12** in the presence of an excess amount of Na^+ or K^+. Because the titration of **3** in the two-phase solvent system ($CHCl_3$/50 mM HEPES (pH 7.4, $I = 0.1$ ($NaNO_3$ or KNO_3)) with **12** at 37 °C were unsuccessful due to the low solubility of **3** in aqueous solution, the UV/Vis titrations were successfully carried out in DMSO/50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$ or KNO_3)) (4/96) at 37 °C. In these experiments, Na^+ and K^+ were added to the buffer at high concentration (0.05 M) to check their effect on the complexation of **3** with **12**. As shown in Figure 2a and Figure 2b, **3** (40 μM) has absorption maxima (λ_{max}) at 287 nm, which increased upon the addition of **12** and reached a plateau at a 1:1 ratio. It should be noted that the complexation of **3** (and **12**) with K^+ and Na^+ is supposed to be very weak in a polar solvent system such as DMSO/H_2O (4/96). These results suggest that the 2:2 complexation of **3** and **12** is not negligibly affected by K^+ and Na^+ under these conditions.

The addition of Cu^{2+} to **13b** and **13c** (formed from **3** and **12** in a 1:1 ratio in the presence of Na^+ and K^+, respectively) induced a red shift from 287 nm to 307 nm, which reached a plateau at [**13b** or **c**]:[Cu^{2+}] = 1:2, as shown in Figure 3a and Figure 3b, suggesting the quantitative formation of the 2:2:2 supermolecules **14b** and **14c** at μM order concentrations in the presence of Na^+ or K^+ in DMSO/50 mM HEPES buffer (pH 7.4 with $I = 0.1$ ($NaNO_3$ or KNO_3)) (4/96) at 37 °C.[17]

Hydrolysis of MNP by 2:2:2 Complexes in a Two-Phase Solvent System

The hydrolysis of MNP (100 μM) by **14** (20 μM in the total solution including the aqueous phase and the $CHCl_3$ phase), which was formed by the self-assembly of **3**, **12**, and Cu^{2+}, was conducted in a two-phase solvent system ($CHCl_3$/50 mM HEPES buffer (pH 7.4) with $I = 0.1$ ($NaNO_3$ or KNO_3)) (2/8) at 37 °C. In such system, it is very likely that Na^+ and K^+ are extracted from the aqueous phase to the organic layer by complexation with the azacrown ether moiety of **12** to form **14** (Scheme 1a and Scheme 3). As shown in Figure 4a, the hydrolysis of MNP (100 μM) in the presence of **10a** and **14c** (20 μM) proceeded to a similar extent and the supermolecule **14a–b** (with Li^+ and Na^+) showed a somewhat lower activity than those of **10a** and **14c** (with K^+). Next, the hydrolysis of MNP by **10a**, **14a**, **14b** and **14c** (20 μM) was carried out at [MNP] = 1000 μM. As shown in Figure 4b, **14c**, whose crown ether parts are supposed to be complexed with K^+ in the organic phase, exhibited a higher

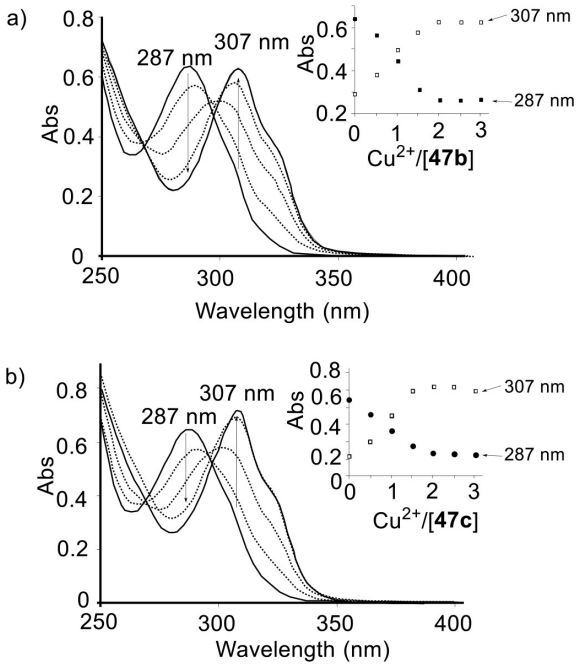


Figure 3. (a) UV/Vis titrations of **13b** (from **3** + **12** + Na^+) (20 μM) upon the addition of Cu^{2+} in DMSO/50 mM HEPES buffer (pH 7.4 with $I = 0.1$ ($NaNO_3$)) (4/96) at 37 °C. (b) UV/Vis titrations of **13c** (from 3 + **12** + K^+) (20 μM) upon the addition of Cu^{2+} in DMSO/50 mM HEPES buffer (pH 7.4 with $I = 0.1$ (KNO_3)) (4/96) at 37 °C.

activity than **10a** and **14a**–**b**. It was also found that the reactivity of **10a** in the presence of 10 eq. (against **10a**) of 1-aza-18-crown-6 ether was almost same as that of **10a** alone in the two-phase solvent system containing an excess amount of KNO_3 (0.05 M) (Figure 4b). Therefore, we concluded that higher MNP hydrolysis reactivity of **14c** than other supramolecular complexes is due to the intramolecular effect of the crown-K^+ units of **14c**. As shown in Figure 4c, the hydrolysis of MNP by **14c** (20 μM) at [MNP] = 100, 200, 500, 800 and 1000 μM (namely, at [**14c**]/[MNP] = 20 %, 10 %, 4 %, 2.5 % and 2 %) gives more than 20 μM of the product (indicated with a dashed straight line in Figure 4c), suggesting that **14c** functions as a catalyst for the hydrolysis of MNP.

The hydrolysis of MNP (100 μM) by supermolecules **14d**–**f** (20 μM) in the presence of Rb^+, Mg^{2+} and La^{3+} (**14d**, **14e** and **14f**, respectively) was also carried out ($RbNO_3$, $Mg(OH)_2$ and $La(NO_3)_3$ were used as the metal sources) at [MNP] = 100, 200, 500, 800 and 1000 μM and typical results at [MNP] = 1000 μM are summarized in Figure 5. Once again, the higher activity of K^+-**14** complex (**14c**) can be explained by higher stability and selectivity of the 18-crown-6 ether-K^+ complex unit than those with other metals in the organic layer.[15]

Although there are several reports on the complexation of crown ether derivatives with alkaline earth metals (e.g., Mg^{2+} and Ca^{2+}) and lanthanides (e.g., Y^{3+}, La^{3+}, Eu^{3+}, etc.),[16] the CTN values of **14e** and **14f**, which possibly contain Mg^{2+} and La^{3+}, were nearly same as or lower than that of **14c** (K^+ complex). As displayed in Figure 6, the CTN value for **14c** is 4.7 at [MNP] = 1000 μM, while the CTNs of **14a** is lower and those of **14b** and **14d** are similar to that of our previously reported complex **10a** (CTN ~ 4).

Michaelis-Menten Kinetics for the Hydrolysis of MNP by 14a-f in the Two-Phase Solvent System

The results for the hydrolysis of MNP by the supramolecular phosphatases at [MNP] = 100, 200, 500, 800, and 1000 μM (in the total solution) were analyzed based on Michaelis-Menten kinetics, as described in our previous reports.[10r,11–13] The reciprocal values for the concentrations of MNP and the rate of hydrolysis (V_o) in the total solution of the two-phase solvent system were plotted to calculate V_{max} (the maximum velocity for the formation of NP from MNP catalyzed by **14**, μM/min) and K_m (Michaelis constant, μM). Based on the Lineweaver-Burk plots shown in Figure 7, the K_m, V_{max} and k_{cat} (rate constants for the catalytic conversion of the substrate into the product, as defined by eq. (1))[10r] values for the supramolecular phosphatases were determined as summarized in Table 1. These values clearly imply that **14c** has the highest V_{max} ($(7.9 \pm 0.4) \times 10^{-2}$ μM/min), k_{cat} ($(4.0 \pm 0.2) \times 10^{-3}$ min^{-1}) and CTN (4.7) among **14a**–**f** (see also Figure 4b), possibly due to the positive effect of the crown-K^+ complex unit on MNP hydrolysis.

$$k_{cat} = V_{max}/[\text{Catalyst}] \quad (1)$$

The hydrolysis of MNP by **14b** and **14c** in the presence of HPO_4^{2-} was examined in order to determine the K_i values (inhibitory constants) of HPO_4^{2-}, as listed in Table 1, because it is well described that natural AP (competitive inhibition)[18] and our artificial AP models (competitive or mixed type inhibition)[11–13] are inhibited by HPO_4^{2-}. The K_i values for **14b** and **14c** were determined to be *ca.* 40 μM and *ca.* 97 μM (entries 6 and 7), respectively, possibly in a mixed-type manner. It should be mentioned that the K_i value of **14c** (*ca.* 97 μM) is greater than those of **8a**, **9a**, and **10a** (*ca.* 15 μM, *ca.* 36 μM and *ca.* 80 μM, respectively), indicating that the K^+ ion would assist the release of inorganic phosphate from the $Cu_2(\mu\text{-}OH)_2$ core, although its mechanism is yet to be studied.

We previously reported that the K_m/K_i values are good parameters to evaluate the catalytic activity of the supramolecular phosphatases (smaller K_m/K_i value is more favorable).[13] For example, the K_m/K_i values (4.8 ~ 5.2) for **10a** and **14b**–**c** (entries 4, 6 and 7 in Table 1) are close to that for AP (*ca.* 2.3) (entry 1 in Table 1), and smaller than those for **8a** (*ca.* 27) and **9a** (*ca.* 36), whose CTN are 0.4 ~ 1 (entries 1 and 3). The V_{max} and CTNs of **14e** (with Mg^{2+}) and **14f** (with La^{3+}) for the hydrolysis of MNP were found to be smaller than those of **14c** (entries 9 and 10 in Table 1). These data allowed us to conclude that **14c** having crown-K^+ complex has highest phosphatase activity among supramolecular complexes tested in this study.

Conclusion

We report on the design, and synthesis of Bar building block functionalized with 1-aza-18-crown ether moiety (**12**) for the construction of supramolecular phosphatases **14** containing alkaline metals and other metals in the reaction medium by means of the self-assembly with **3** (Zn_2L^3) and Cu^{2+} in a two-phase solvent system to mimic the structure of the active site of AP. The hydrolysis of MNP by the supramolecular complexes **14a**–**f** follows Michaelis-Menten kinetics in the two-phase

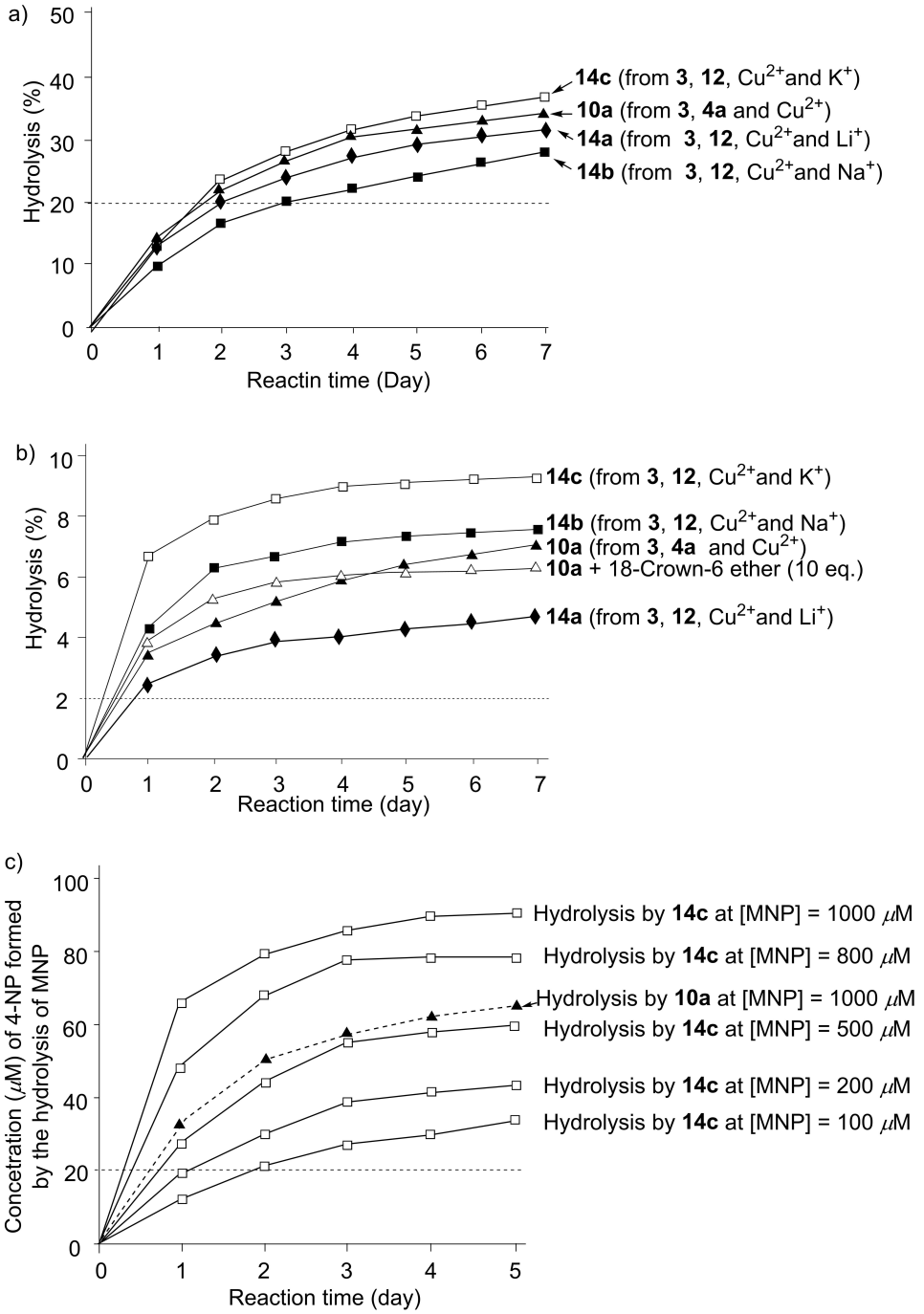


Figure 4. (a) Hydrolysis (%) of MNP (100 μM in total solution) by **14a** (20 μM) in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($LiNO_3$)), **14b** (20 μM) in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$)), **14c** (20 μM) in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3)) and **10a** (20 μM). (b) Hydrolysis (%) of MNP (1000 μM in total solution) by **10a** (20 μM), **10a** (20 μM) + 1-aza-18-crown-6 ether (200 μM)) in 50 mM HEPES buffer (pH 7.4 with $I = 0.1$ (KNO_3)), **14a** (20 μM) in $CHCl_3$/ 50 mM HEPES (pH 7.4 with $I = 0.1$ ($LiNO_3$)), **14b** (20 μM) in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$)), and **14c** (20 μM) $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3)). (c) Change in the concentration of 4-NP, which is produced by the hydrolysis of MNP promoted by **14c** (20 μM) at [MNP] = 100 μM, 200 μM, 500 μM, 800 μM and 1000 μM (solid line with open squares) and change in the concentration of 4-NP at [MNP] = 1000 μM produced by **10a** (20 μM) (dashed line with filled triangles) in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3)).

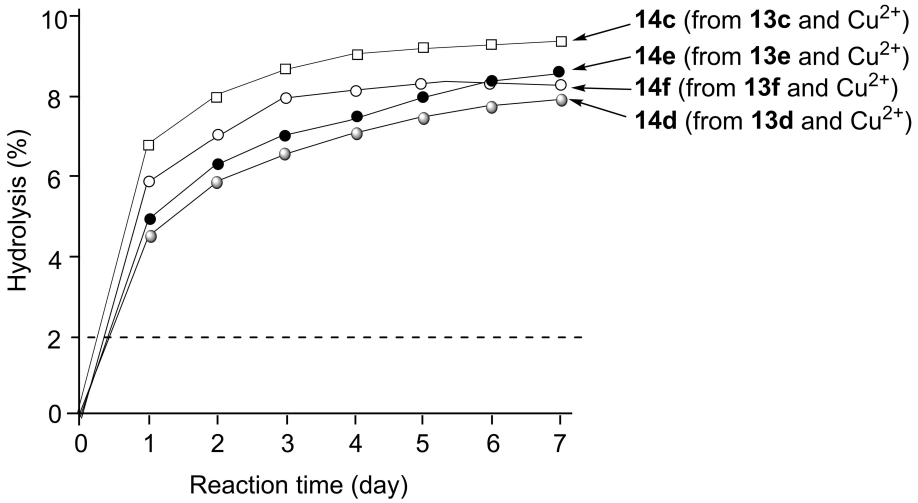


Figure 5. Hydrolysis (%) of MNP (1000 μM in the total solution) by **14c** (20 μM) in the presence of 0.05 M K^+ in 50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3), **14d** (20 μM) in the presence of 0.05 M Rb^+ in 50 mM HEPES (pH 7.4 with $I = 0.1$ ($RbNO_3$), **14e** (20 μM) and **14f** (20 μM) in 50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$)) at 37 °C, 4 eq. of $Mg(OH)_2$ and $La(NO_3)_3$ was added in the respective solution as source of Mg^{2+} and La^{3+}.

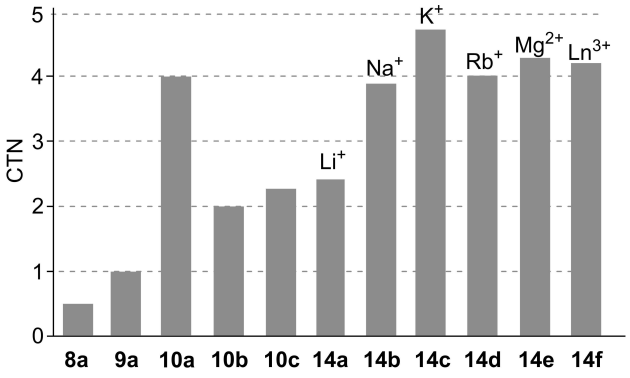


Figure 6. Comparison of catalytic turnover numbers (CTNs) of **8a**, **9a**, **10a**, **10b**, **10c** and **14a–f** (20 μM in total solution) at [MNP] = 1000 μM in $CHCl_3$/50 mM HEPES buffer (pH 7.4) with $I = 0.1$ ($LiNO_3$, $NaNO_3$, KNO_3 or $RbNO_3$) (2/8) at 37 °C.

solvent system in this study. The values of K_m/K_i ratio of **14b–c** (4.9–5.2) are also comparable to our previously reported catalytic complex **10a** (4.8). In comparison with the MNP hydrolysis by **9** and **10** in the two-phase system, **14c** comprised of two Cu^{2+} ions and possibly one K^+ ion in its active site proved to be more efficient in terms of a faster and higher rate of catalysis than **9** and **10** that contained no crown ether unit. We therefore conclude that the unprecedented 2:2:2:4 complexes of the bis(Zn^{2+}-cyclen) complex **3** (Zn_2L^3), the Bar building block equipped with the 18-crown-6 ether (**12**), Cu^{2+}, and alkaline metal cations such as K^+ are constructed in the two-phase solvent system ($CHCl_3/H_2O$) and that the catalytic phosphatase activity is improved by the functionalization of Bar unit (eventually, functionalization of the supramolecular phosphatase) with the crown ether-K^+ complex as a mimic of the Mg^{2+} in the catalytic site of AP. We believe that this type of functionalization of supramolecular complexes opens a strategy for assessing their catalytic activity for the hydrolysis of MNP in a two-phase solvent system. These findings should be useful for the design of more efficient catalysts with reference to biochemistry including enzymatic reactions.

Experimental Section

General Information

All reagents and solvents were of the highest commercial quality and were used without further purification, unless otherwise noted. *N*,*N*-Dimethylformamide (DMF) was obtained by distillation from calcium hydride. MNP was purchased from Nacalai Tesque (Japan). All aqueous solutions were prepared using deionized and distilled water. The Good's buffer reagents (Dojindo, pK_a at 20 °C) were obtained from commercial sources: HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl))ethanesulfonic acid, pK_a = 7.6). For the measurement of UV/Vis spectra, $CHCl_3$ and DMSO were purchased from Nacalai Tesque (Japan). UV/Vis spectra were recorded on a JASCO V-550 or JASCO V-630 spectrophotometer with quartz cuvettes (path length: 10 mm). IR spectra were recorded on a Perkin-Elmer attenuated total reflectance (ATR)-IR spectrometer 100 at room temperature. ^{1}H- (300 MHz) and ^{13}C– (100 MHz) NMR spectra at 25 ± 0.1 °C were recorded on a JEOL Always 300 spectrometer. Mass spectra were recorded on a JEOL JMS-700 and Varian 910-MS spectrometer. Thin-layer chromatography (TLC) and alumina gel column chromatography was performed using Merck Silica gel 60 F254 plate and Wako pure chemical Alumina Gel for chromatography, respectively.

*5,5-Bis(3-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) propyl)-pyrimidine2,4,6-(1*H*,3*H*,5*H*)-trione (12)*

To a solution of 1-aza-18-crown-6 ether (0.1 g, 0.38 mmol) and triethylamine (0.04 g, 0.38 mmol) in distilled DMF (0.6 ml), compound **18**[12b] (0.1 g, 0.17 mmol) was added at room temperature. The reaction mixture was stirred at 70 °C for 6 h under an argon atmosphere. After evaporating the reaction mixture to dryness, the resulting residue was purified by alumina gel column chromatography ($CHCl_3$/MeOH = 25/1) to give **12** as a colorless oil. IR (ATR) cm^{-1}: 2860, 1723, 1698, 1408, 1350, 1109, 943, 838, 499. ^{1}H-NMR

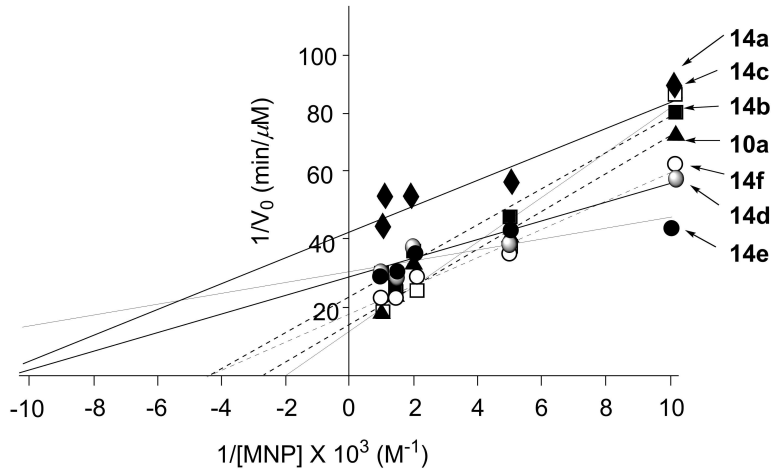


Figure 7. Lineweaver-Burk plots for the hydrolysis of MNP catalyzed by **14a** (closed diamond with solid line), **14b** (closed square with dashed line), **14c** (open squares with solid line), **14d** (gray circles with solid line), **14e** (closed circles with solid line), **14f** (open circles with dashed line), and **10a** (closed triangles with dashed line).

Table 1. Typical kinetic parameters for the hydrolysis of MNP by **8a** and AP in a single-phase solvent system (10 mM HEPES buffer (pH 7.4) with $I = 0.1$ ($NaNO_3$)) at 37 °C, and by **9a**, **10a** and **14a–f** in a two-phase solvent system ($CHCl_3$/50 mM HEPES buffer (pH 7.4) with $I = 0.1$ ($LiNO_3$, $NaNO_3$ or KNO_3 or $RbNO_3$) (2/8)) at 37 °C.

Entry	Cat.[a]	M^{n+}	V_{max} [μMmin^{-1}]	K_m [μM]	k_{cat} [min^{-1}][b]	K_i [μM]	K_m/K_i	CTN[c]
1	**8a**[d]	-	$(8.9 \pm 0.2) \times 10^{-2}$ [d]	$(4.1 \pm 0.3) \times 10^{2}$ [d]	$(8.9 \pm 0.2) \times 10^{-4}$ [d]	*ca.*15 (mixed-type)	*ca.* 27	0.4
2	**AP**[d]	-	1.3 ± 0.1[e]	7 ± 4[e]	$(2.4 \pm 0.2) \times 10^{3}$[e]	3 ± 1 (competitive)	*ca.* 2.3	$>10^3$[f]
3	**9a**[g]	-	$(1.4 \pm 0.4) \times 10^{-2}$[g]	$(5.4 \pm 0.5) \times 10^{2}$[g]	$(7.0 \pm 2.0) \times 10^{-4}$[g]	*ca.*15 (competitive)	*ca.* 36	1.0
4	**10a**[h]	-	$(6.8 \pm 0.3) \times 10^{-2}$[h]	$(3.8 \pm 0.2) \times 10^{2}$[h]	$(3.4 \pm 0.2) \times 10^{-3}$[h]	*ca.*80[h] (mixed-type)	*ca.* 4.8 [h]	~4
5	**14a**[i]	Li^+	$(2.2 \pm 0.3) \times 10^{-2}$	90 ± 6	$(1.1 \pm 0.2) \times 10^{-3}$	n.d[m]	n.d.[m]	2.4
6	**14b**[j]	Na^+	$(4.4 \pm 0.2) \times 10^{-2}$	$(2.3 \pm 0.3) \times 10^{2}$	$(2.2 \pm 0.1) \times 10^{-3}$	*ca.*40 (mixed-type)	*ca.* 4.9	3.9
7	**14c**[k]	K^+	$(7.9 \pm 0.4) \times 10^{-2}$	$(5.9 \pm 0.3) \times 10^{2}$	$(4.0 \pm 0.2) \times 10^{-3}$	*ca.*97 (mixed-type)	*ca.* 5.2	4.7
8	**14d**[l]	Rb^+	$(3.5 \pm 0.2) \times 10^{-2}$	94 ± 2	$(1.8 \pm 0.1) \times 10^{-3}$	n.d.[m]	n.d.[m]	4
9	**14e**[j]	Mg^{2+}	$(3.3 \pm 0.4) \times 10^{-2}$	51 ± 3	$(1.7 \pm 0.2) \times 10^{-3}$	n.d.[m]	n.d.[m]	4.3
10	**14f**[j]	Ln^{3+}	$(5.5 \pm 0.3) \times 10^{-2}$	$(2.2 \pm 0.2) \times 10^{2}$	$(2.8 \pm 0.2) \times 10^{-3}$	n.d.[m]	n.d.[m]	4.2

[a] Cat = catalyst. [b] Calculated by eq. (1) in the text. [c] Catalytic turnover numbers determined at [MNP] = 1000 μM (see Figure 6). [d] From ref. 11 (in a single aqueous solution (10 mM HEPES, pH 7.4 with $I = 0.1$ ($NaNO_3$)). [e] From ref. 10r (in a single aqueous solution (10 mM HEPES, pH 7.4 with $I = 0.1$ ($NaNO_3$)). [f] Calculated from data in ref. 10r. [g] From ref. 12a. [h] From ref. 13. [i] Determined in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($LiNO_3$)) (2/8). [j] Determined in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($NaNO_3$)) (2/8). [k] Determined in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ (KNO_3)) (2/8). [l] Determined in $CHCl_3$/50 mM HEPES (pH 7.4 with $I = 0.1$ ($RbNO_3$)) (2/8). [m] Not determined.

(300 MHz, CD_3OD/TMS) δ: 3.64–3.57 (m, 40H), 2.75(t, 8H, $J = 5.5$H), 2.52 (t, 4H, $J = 7.2$H), 1.92–1.87 (m, 4H), 1.44–1.33 (m, 4H). ^{13}C-NMR (75 MHz, CD_3OD/TMS) δ: 175.8, 152.5, 71.7, 71.6, 71.3, 71.2, 70.1, 56.6, 56.5, 54.9, 37.6, 23.5. HRMS (ESI-MS) m/z: 735.43 (Calcd. for $C_{34}H_{62}N_4O_{13}$ $[M+H]^+$: 735.43).

Hydrolysis of MNP

The hydrolysis of MNP was carried out in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ ($NaNO_3$) (2/8) (for **14b**, **14e** and **14f**). For the MNP hydrolysis with **14e** and **14f**, **14e** was prepared at $[Mg(OH)_2] = 80$ μM and $[NaNO_3] = 0.05$ M and **14f** was prepared at $[La(NO_3)_3] = 80$ μM and $[NaNO_3] = 0.05$ M, due to low solubility of Mg^{2+} and La^{3+} ions. The hydrolysis of MNP was carried out in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ ($LiNO_3$) (2/8) (for **14a**) and in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ (KNO_3) (2/8) (for **14c**) and in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ ($RbNO_3$) (2/8) (for **14d**) at 37 °C. Stock solutions of **12** (6.0 mM in H_2O), and $Cu(ClO_4)_2 \cdot 6H_2O$ (6 mM in H_2O) were used for the preparation of sample solutions of **14** in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ ($LiNO_3$, $NaNO_3$, KNO_3, or $RbNO_3$) (2/8) (total volume = 3.0 mL). A stock aqueous solution of MNP (20 mM) was used for the hydrolysis reaction. Prior to the hydrolysis of MNP in the two-phase solvent system, the reaction mixtures of **3**, **12**, and Cu^{2+} in $CHCl_3$/50 mM HEPES (pH 7.4) with $I = 0.1$ ($LiNO_3$, $NaNO_3$, KNO_3, or $RbNO_3$) (2/8) were incubated overnight at 37 °C using a shaking heat controlled incubator (shaking speed: 150 rpm) (BioShaker BR-23FP, TAITEC, Japan) to form **14a–f** *in situ*, and the aqueous solution of MNP was then added. All of the hydrolysis experiments were performed in triplicate. The yields of the hydrolysis product from MNP in the presence of the supramolecular complexes were calculated based on the increase in the absorption of the released 4-nitrophenol (NP) at 400 nm ([NP

produced in the presence of the supramolecular complex]-[NP produced in the absence of the supramolecular complex]) (ε_{400} value of NP is 1.35×10^4 $M^{-1}\cdot cm^{-1}$ at pH 7.4)[10r] in aqueous layer. The partition ratio of NP (79% in aqueous solution), which had been determined in a previous report[12,13] was used for the calculation of the yields in the $CHCl_3/H_2O$ system.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Supramolecular catalysts · Hydrolysis · Phosphate monoesters · Phosphatase · Zinc · Copper · Potassium

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