

A Semi-Flexible Tetrahydrazone Macrocyclic for Binding of Pyrophosphate and Smaller Anions

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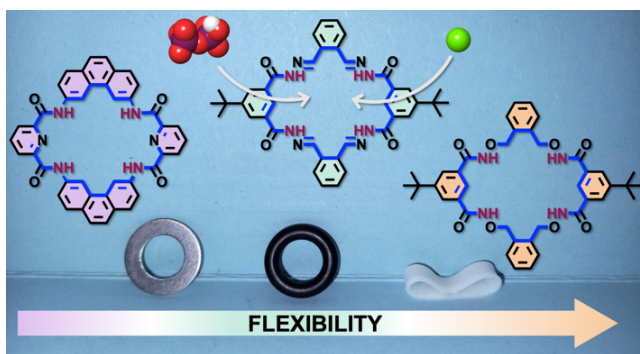
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ABSTRACT: Macrocyclization has proven to be a useful design strategy in the development of efficient anion receptors. In addition to ring size, the overall preorganization by structural rigidity is of key importance. To explore this in the context of developing an efficient pyrophosphate receptor, three macrocycles featuring a 26-membered interior ring size and similar H-bonding motifs have been synthesized and their anion binding ability investigated. Computational studies and NMR data showed different degrees of preorganization as a result of differences in flexibility. The interaction of the three macrocycles with chloride, dihydrogen phosphate, and dihydrogen pyrophosphate was investigated in solution by NMR and UV-Vis spectroscopy, and in the solid state by X-ray crystallography. The tetrahydrazone based macrocycle featuring intermediate flexibility exhibited the best affinity for all three anions investigated. Our results suggest that in addition to the proper preorganization of binding groups in a macrocycle a certain degree of flexibility is also required for an optimal affinity with the target guest.



Introduction

Anion recognition is at the basis of processes such as sensing or transmembrane transport.¹⁻⁴ The recognition of phosphate species is of particular interest in the biological field. Various phosphate species play important roles in a wide range of biological processes, including encoding of genes, energy storage, and as a crucial building block for bones and teeth.⁵ Furthermore, phosphate species can also be linked to diseases such as hyperphosphatemia, characterized by increased levels of phosphate in the blood,⁶ or pseudogout, a form of arthritis caused by calcium pyrophosphate dihydrate (CPPD) deposits.^{7,8} Pyrophosphate in serum is formed primarily by the hydrolysis of ATP, while it is in turn hydrolyzed to inorganic phosphate. Pyrophosphate is essential to control mineralization, avoiding undesired calcium phosphate (hydroxyapatite) formation, while growing CPPD crystals at elevated pyrophosphate levels.⁷⁻⁹ As a result of the importance of phosphate species, receptors capable of effectively binding, sensing, or transporting these species are of great interest.¹⁰⁻¹⁴

One of the most common approaches for the development of phosphate and pyrophosphate receptors has been the use of NH- or OH-based H-bond donors, mimicking the way that natural receptors achieve phosphate

recognition.¹⁵⁻¹⁷ It is well established that the proper preorganization of the molecular structure contributes to the affinity.¹⁸⁻²⁰ A macrocyclic approach is therefore often used. Size complementarity between receptor and substrate was shown early on by Dietrich *et al.* to have a significant effect on the stability of cryptand anion complexes,²¹ and numerous other contributions have ensued this seminal work. Focusing on neutral tetralactam-based macrocycles as anion receptors, Chmielewski and Jurczak have shown that the size of macrocyclic receptors plays an essential role in both the selectivity and strength of receptor-anion interactions.^{22,23} Their work also showed that pyridyl N-atoms in dipicolinic bisamides contribute to the preorganization of the amides for anion binding, resulting in significant increases in binding affinities compared to isophthalamides.²² The comparison of the 18-membered tetralactam receptor with ethylene spacers of Szumna and Jurczak²⁴ with its aromatic and rigid counterpart prepared by Smith and co-workers²⁵ seems to support the notion that more rigid macrocycles lead to higher anion affinities. Similarly, Flood and co-workers obtained higher binding affinities with more rigid triazole based anion receptors compared to their more flexible counterparts.²⁶ On the other hand, examples can also be found where an increase in flexibility within a macrocyclic structure can be beneficial for binding, such as the

work carried out by Glas *et al.* in which they investigated the effect of conformational flexibility of two similar peptide based macrocyclic receptor on the stability of the corresponding macrocycle-ligand complexes. The most flexible of the two showed the highest affinity.²⁷

With this notion of the importance of size, flexibility, and preorganization in mind, we set out to develop a series of macrocyclic receptors for dihydrogen pyrophosphate ($\text{H}_2\text{P}_2\text{O}_7^{2-}$), exhibiting identical macrocyclic ring sizes and possessing in all cases four NH-based hydrogen bond donors. Here, we describe the design, synthesis, and characterization of macrocycles **1**, **2**, and **3** (Figure 1) and their ability to bind $\text{H}_2\text{P}_2\text{O}_7^{2-}$, as well as the smaller anions H_2PO_4^- and Cl^- in solution and in the solid state.

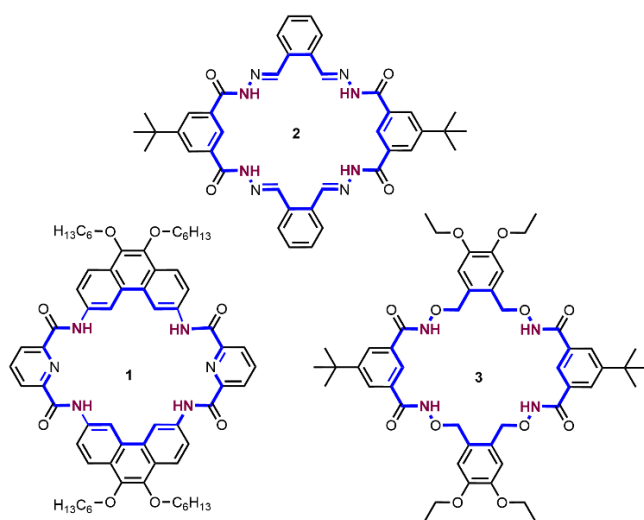


Figure 1. The three macrocycles synthesized and investigated in this work, all with an interior macrocycle size of 26 atoms (blue) and four NH groups as H-bond donors (dark red).

Results and discussion

Macrocycle design and molecular modelling. Previously reported neutral macrocyclic receptors for $\text{H}_2\text{P}_2\text{O}_7^{2-}$ have utilized 24- and 32-membered molecular scaffolds to achieve anion binding.^{28,29} With the help of computational studies we found that 26-membered macrocycles would allow the design of D_{2h} symmetric structures with binding cavities showing good size complementarity for $\text{H}_2\text{P}_2\text{O}_7^{2-}$.

Three different macrocycles with an increasing degree of flexibility, but all with 26 atoms in the macrocyclic ring were designed as anion receptors to find the optimal compromise between preorganization and flexibility (Figure 1). Macrocycle **1** was designed to be the most rigid of the three. It employs dipicolinic bisamides, which are known to prefer a *syn-syn* configuration due to interactions of the amide NH and the lone pair on the pyridyl N,^{22,23,30,31} as well as phenanthrene spacers to give rigidity and the desired appropriate macrocyclic ring size. This in turn was envisaged to result in preorganization of the amides to accommodate $\text{H}_2\text{P}_2\text{O}_7^{2-}$ in the binding cavity. Macrocycle **2** was designed to be more flexible, replacing the dipicolinic bisamide motif with isophthalic acylhydrazones. This could potentially also lead to interactions from the CH proton of the isophthalic subunit between the two acylhydrazones, which has been reported to be

able to participate in anion binding.^{25,32,33} Macrocycle **3**, employing bis-N-(benzyloxy)isophthalamides, was designed to be the most flexible. The *n*-hexyl and ethyl ether groups of **1** and **3** respectively, as well as *tert*-butyl groups of **2** and **3** are present to enhance the solubility of the macrocycles in organic solvents as required for anion binding studies.

Macrocycles **1-3** have four NH H-bond donating groups at the same positions in the macrocycles. However, the groups of these three macrocycles vary from acylhydrazones to (N-alkoxy)amides, which could potentially lead to differences in binding between the three macrocycles. To try and gauge the influence of these H-bond donors on anion affinity, both literature data^{34,35} on anion binding of these functional groups as well as their estimated pK_a were considered (see SI for details). It appears that the available anion binding data as well as the pK_a values of the moieties in question are not too different and thus not likely to be the main source of observed differences.

Computational studies using a semi-empirical PM7 approach in MOPAC were carried out for each of the three receptors without guest, with $\text{H}_2\text{P}_2\text{O}_7^{2-}$, and with the much smaller Cl^- anion to gain insights in the differences in the conformation of the receptors. In the absence of any guest (Figure 2a-c), macrocycle **1** adopts a preferred conformation with all the NHs pointing into the cavity, suggesting a good preorganization for $\text{H}_2\text{P}_2\text{O}_7^{2-}$ binding. Macrocycle **2** adopts a preferred conformation with two of the NH groups pointing into and the other two pointing out the cavity. Macrocycle **3** exhibits intramolecular H-bonding, with two of the NH protons interacting with carbonyl groups pointing towards the interior of the cavity and the other two NH protons pointing outwards.

In the presence of $\text{H}_2\text{P}_2\text{O}_7^{2-}$, all three macrocycles were able to adopt conformations in which all four H-bond donors are interacting with the guest (Figure 2d-f), however with different NH to O bond lengths: the NH to O distances fall within the range of 1.47-1.80 Å with averages of 1.64 Å and 1.70 Å for macrocycles **2** and **3** respectively, whereas the average H-bond length of macrocycle **1** is 1.88 Å. Macrocycles **2** and **3** can furthermore adapt their conformation to have all four NH groups interacting with a smaller Cl^- anion (Figure 2h and 2i, NH to Cl distances of 2.19-2.35 Å), while the more rigid macrocycle **1** is unable to utilize all four NH groups to interact with the Cl^- anion, which is thus located at one of the two dipicolinic bisamide sites (Figure 2g, NH to Cl distances of 1.99 and 2.03 Å).

These computational studies confirm that macrocycle **1** is well preorganized for $\text{H}_2\text{P}_2\text{O}_7^{2-}$ binding, with all NH groups pointing into the cavity even in absence of a guest. However, its rigidity hinders it from forming four H-bonds to the small Cl^- anion. Macrocycle **2** is less preorganized than **1**, with only two NH groups pointing into the cavity in its lowest energy conformer, but it can adapt its conformation to bind $\text{H}_2\text{P}_2\text{O}_7^{2-}$ or Cl^- with all NH groups. Macrocycle **3** is least preorganized for anion binding with intramolecular H-bonds closing the cavity in absence of a guest, but it has a good adaptability to anionic guests of different sizes. Note that only NH protons are shown in Figure 2 but that CH protons pointing into the cavity could also contribute to anion binding.

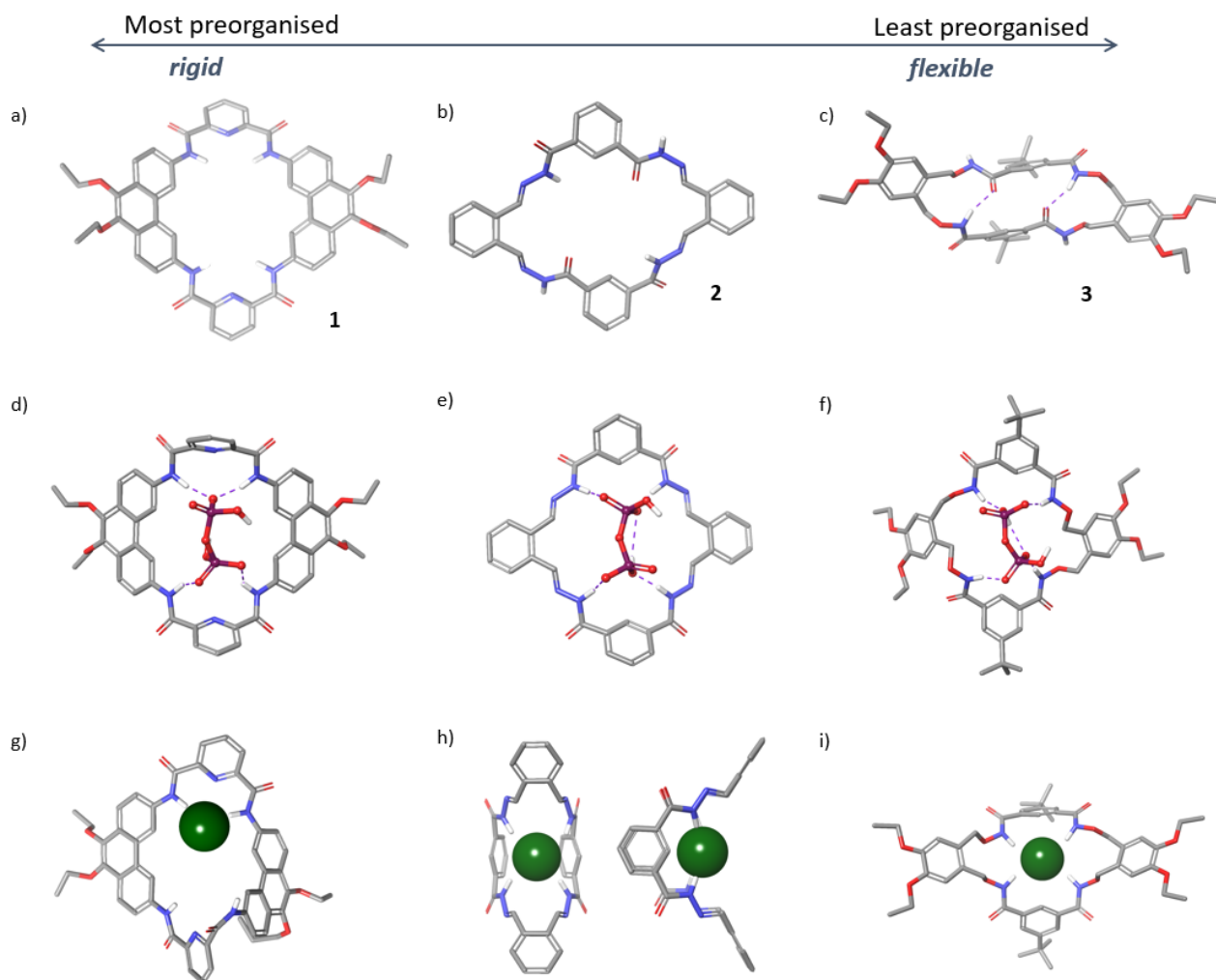
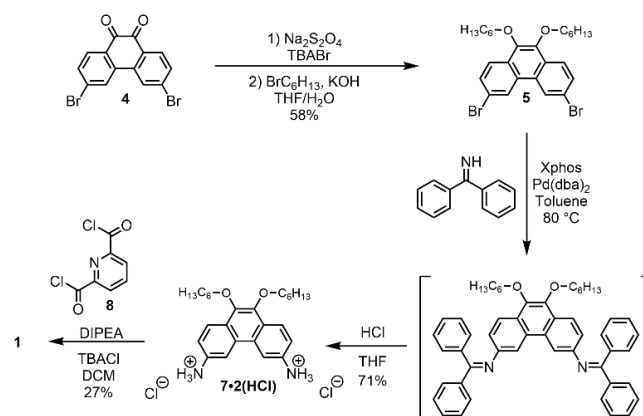


Figure 2. Molecular models (PM7 optimized geometries, vacuum) of lowest energy conformations of macrocycles **1-3** without anions (a-c), with $\text{H}_2\text{P}_2\text{O}_7^{2-}$ (d-f) and Cl^- (g-i).

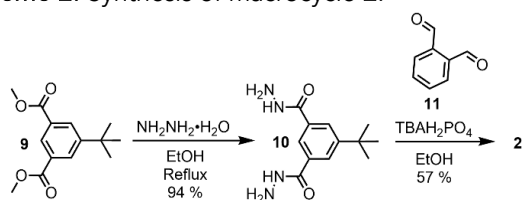
Synthesis. Macrocycle **1** was synthesized by the condensation of diacylchloride **8**³⁶ with 3,6-diamino-9,10-dihexoxyphenanthrene **7** (Scheme 1). Diamino-phenanthrenes with this substitution pattern have not been reported to date but could be useful building blocks in organic synthesis for various applications. While 3,6-diamino-9,10-dihexoxyphenanthrene **7** could not be obtained in pure form as the free base due to its propensity to decompose, a synthesis route to obtain the HCl salt of **7** in three steps from commercially available 3,6-dibromophenanthrene-9,10-dione **4**³⁷ was developed. This route starts by reductive etherification of **4** to get 3,6-dibromo-9,10-dihexoxyphenanthrene **5**,³⁸ followed by a Buchwald-Hartwig cross coupling with benzophenone imine **6** and subsequent acid hydrolysis of the formed bisimine. The reaction between the HCl salt of phenanthrediamine **7**·2(HCl) and diacylchloride **8** in the presence of DIPEA as base affords the desired macrocycle in around 10% yield. However, addition of 45 equivalents of tetrabutylammonium chloride (TBACl) improved the yield significantly and facilitated purification by column chromatography due to the better solubility of **1** in the presence of TBACl. Additional optimization steps such as slow addition of reagents and performing the reaction in the dark and under an argon atmosphere to try and prevent decomposition of the free base of **7**·2(HCl) ultimately improved the yield to 27%.

Scheme 1. Synthesis of macrocycle **1**.



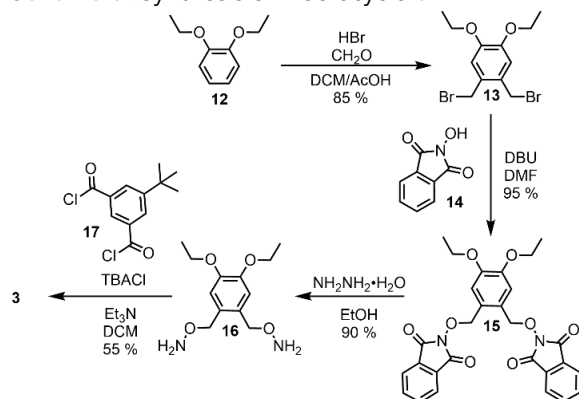
Macrocycle **2** was synthesized via precursor **9**³⁹ by the condensation of bishydrazide **10**⁴⁰ with phthalaldehyde **11** in the presence of TBAH_2PO_4 in 57% yield (Scheme 2). The H_2PO_4^- template proved to be essential since, when absent, the macrocycle was obtained as a mixture with larger macrocycles and oligomers which could not be separated, whereas the presence of TBAH_2PO_4 caused the desired macrocycle to selectively precipitate from solution.

Scheme 2. Synthesis of macrocycle 2.



Finally, macrocycle **3** was synthesized in a similar manner as macrocycle **1** by condensation of *bis*-*O*-hydroxylamine **16** with diacylchloride **17**⁴¹ (Scheme 3). The *bis*-*O*-hydroxylamine was obtained in three steps by bromomethylation of 1,2-diethoxybenzene **12**, followed by nucleophilic substitution by *N*-hydroxyphthalimide **14**⁴² on **13**⁴³ and subsequent hydrazinolysis. Notably, for this macrocyclization, the rate of addition had little effect on the yield of the reaction and increasing the equivalents of TBACl beyond 10 only had a negligible effect on the yield. Moreover, the addition of other templates such as TBAH₂PO₄ or TBA₂H₂P₂O₇ gave lower yields compared to the 55% yield obtained with TBACl.

Scheme 3. Synthesis of macrocycle 3.



Attempts were made at condensing diaminophenanthrene **7** with diacylchloride **17** or glutaryl chloride, at condensing phthalaldehyde **11** with bisacylhydrazines derived from dipicolinic acid or glutaric acid, and at condensing *bis*-*O*-hydroxylamine **16** with diacyl chloride **8**, but the desired macrocycles were either not observed or the purification was unfeasible.

NOE studies. Upon obtaining the three macrocycles, their difference in preorganization and conformational flexibility suggested by modelling was investigated in DMSO-*d*₆ through ¹H NMR 1D-ROESY experiments (Figure 3). When inverting the signal of the amide NH proton (H_c) of macrocycle **1**, a Nuclear Overhauser Effect (NOE) was only observed with phenanthrene proton H_a, consistent with a conformation where all the amide NHs (H_c) are pointing towards the center of the cavity (Figure 2a).

The 1D-ROESY NMR spectrum of macrocycle **2** showed strong NOEs of the NH protons (H_c) with both the azomethine protons H_d and the aromatic protons H_b. A weaker NOE can also be observed with the H_a protons, indicating that the NH can point out of the cavity. The modelling studies showed that macrocycle **2** can adopt conformations with 4, 3, or 2 NH groups pointing into the cavity, conformers which are in fast exchange on the chemical shift time scale, as only a single NH signal is observed in the NMR spectra. Considering that the shortest distances

of NH_c to H_a and to H_b are similar in the conformation of lowest energy derived from the modelling studies (Figure 2b), the larger NOE to H_b implies that in solution on average more NH groups point into the cavity than out of it.

We noted that upon addition of an excess of TBAH₂PO₄ as guest to a solution of macrocycle **2**, the NOE between protons H_c and H_a was no longer observed, while NOEs between H_c and protons H_d and H_b remained visible (Figure S1), suggesting that in the presence of the guest the macrocycle adopts a conformation where all the NH protons are oriented into the binding cavity.

With macrocycle **3** the amide protons H_c exhibit strong NOEs with protons H_a and H_b, consistent with the NHs pointing both inside and outside the cavity, as observed in the model (Figure 2c) as well as in the X-ray crystal structure of the macrocycle (Figure S2), which corroborated the H-bond interactions between two of the carbonyls with the two opposite NH groups.

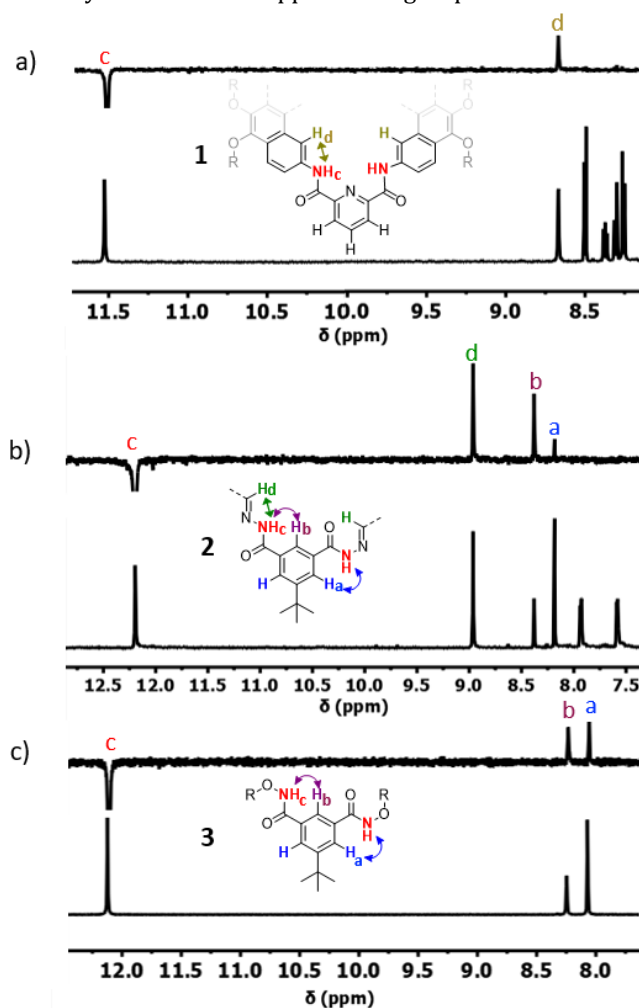


Figure 3. 1D-ROESY (top) and ¹H-NMR spectrum (bottom) of a) macrocycle **1** (600 MHz, DMSO-*d*₆ + 0.5% H₂O, 1D-ROESY mixing time = 200 ms) with interaction observed between proton H_c and H_a; b) macrocycle **2** (600 MHz, DMSO-*d*₆ + 0.5% H₂O, 1D-ROESY mixing time = 200 ms) with interactions observed between proton H_c and H_d, H_b, and H_a; c) macrocycle **3** (400 MHz, DMSO-*d*₆ + 0.5% H₂O, 1D-ROESY mixing time = 250 ms) with interactions observed between proton H_c and H_b and H_a.

Anion binding. The interaction of macrocycles **1-3** with the three anionic guests Cl^- , H_2PO_4^- , and $\text{H}_2\text{P}_2\text{O}_7^{2-}$ was studied through ^1H -NMR titrations with the corresponding tetrabutylammonium salts in $\text{DMSO}-d_6$ with 0.5% H_2O added. For macrocycles **1** and **2**, the ^1H NMR signals of the protons pointing into the cavity showed clear downfield shifts upon addition of TBACl (Figures S3 and S4), while the signals for protons H_c and H_b of macrocycle **3** were shifted less (Figure S5). The displacements of the ^1H NMR signals of the protons pointing into the cavity were fitted with a 1:1 binding model⁴⁴⁻⁴⁶ and $K_{1:1}$ values for Cl^- of $1.2 \cdot 10^2 \text{ M}^{-1}$ and $2.1 \cdot 10^3 \text{ M}^{-1}$ were obtained for **1** and **2** respectively (Table 1).

The intramolecular hydrogen bonding observed for macrocycle **3**, both by modelling (Figure 2c) and by X-ray crystallography (Figure S2), probably explains the poor interaction of this macrocycle with Cl^- . The lower affinity of macrocycle **1** for Cl^- , compared to macrocycle **2**, can be explained by the fact that only one of the dipicolinic bisamide motifs is able to interact with Cl^- (Figure 2g). Thus, the affinity of **1** was found to be within the same order of magnitude, albeit slightly higher, as that observed for dipicolinic bisamides with less preorganization ($10\text{-}47 \text{ M}^{-1}$),⁴⁷ but significantly lower than that of more flexible dipicolinic bisamide macrocycles (1930 M^{-1} , in $\text{DMSO}-d_6$).²² More surprising, however, was the fact that macrocycle **2** exhibits a high affinity for Cl^- compared to, for example, the macrocycles of the same size utilizing four ureas as the binding motifs (up to 500 M^{-1} , in $\text{DMSO}-d_6$),⁴⁸ which could have been expected to provide higher affinities than acylhydrazones.³⁵

During the ^1H -NMR titrations of macrocycle **1** in DMSO (0.5% H_2O) with H_2PO_4^- and $\text{H}_2\text{P}_2\text{O}_7^{2-}$ the signals showed significant broadening after addition of 1-2 equivalents of the salts, preventing a reliable quantification of the binding (Figures S6-7). Additionally, precipitation was observed after addition of 1 equivalent of the salts, further complicating the binding studies. The NH signal of macrocycle **3** also broadened upon addition of H_2PO_4^- and $\text{H}_2\text{P}_2\text{O}_7^{2-}$, while the other signals showed only minimal changes with up to 5 eq. of these salts (Figures S8-9).

In contrast, a set of new signals appeared in the ^1H -NMR spectra of macrocycle **2** upon addition of H_2PO_4^- or $\text{H}_2\text{P}_2\text{O}_7^{2-}$, characteristic of slow exchange on the NMR chemical shift timescale, which allowed a more complete analysis of the binding events (Figure 4). The addition of H_2PO_4^- to **2** led to a gradual decrease of the original H_b signal (8.37 ppm), with a new signal appearing at 8.5 ppm (H_b') during the first part of the titration (up to 1.3 eq. of H_2PO_4^-), which disappeared upon further addition of guest with another signal appearing at 8.9 ppm (H_b''). This behavior is indicative of two binding events, the binding of a first guest anion, followed by formation of a 1:2 complex with H_2PO_4^- . Such 1:2 complexes have been reported previously and their existence has been attributed to the formation of phosphate dimers involving Anti-Electrostatic Hydrogen Bonds (AEHBs).⁴⁹⁻⁵³

During the titration with H_2PO_4^- (Figure 4a), a gradual decrease in the intensity of the H_d azomethine signal at 9.0 ppm was observed concomitant with the appearance of only one new signal H_d' at 9.4 ppm. Signal H_c , only showed some broadening when less than 1 eq. of H_2PO_4^- was added and it is only upon further addition of the guest that

a new signal appeared at 14.1 ppm (H_c'), which is clearly visible in the spectrum with 2.1 eq. of H_2PO_4^- . The variation of the integration of the H_c and H_d signals upon titration are shown in Figure 4b. The integrations suggest that signal H_d' is a superposition of this proton's signals in both the 1:1 complex and the 1:2 complex, whereas signal H_c would be the superposition of the signals arising from the 1:1 complex and the free receptor. The first H_2PO_4^- binding event can thus be observed from the azomethine H_d signals and the second event is reflected by the behavior of the NH_c signals.

Over the course of the titration of macrocycle **2** with $\text{H}_2\text{P}_2\text{O}_7^{2-}$, two distinct complexes were also identified (Figure 4c). Exchange was slow on the NMR chemical shift scale and distinct signals were observed for several protons which could be easily assigned (Figure S12). The ratio between the different complexes could be determined by integration of the signal corresponding to the azomethine H_d or the NH_c (Figure 4d). Crucially, this revealed that, during the first few additions, the amount of new complex formed corresponded to twice the amount of guest added, implying the formation of a 2:1 host to guest complex. Furthermore, the fact that almost complete formation of the second complex takes place once 1 eq. of $\text{H}_2\text{P}_2\text{O}_7^{2-}$ is added implies that the second binding event results in the formation of a strong 1:1 complex.

UV-Vis spectroscopy was used to determine the affinity constants of macrocycles **1** and **2** with TBAH_2PO_4 and $\text{TBAH}_2\text{P}_2\text{O}_7$ in DMSO . Titrations with TBACl were performed for comparative purposes and fitting with a 1:1 binding model gave affinity constants in agreement with those obtained by the ^1H -NMR titrations (see Table 1 and Figures S13-S14). The data from the UV-Vis spectroscopy titrations of **1** with H_2PO_4^- and $\text{H}_2\text{P}_2\text{O}_7^{2-}$ could be fitted with a 1:1 binding model and resulted in affinity constants of $8.9 \cdot 10^2$ and $3.9 \cdot 10^3 \text{ M}^{-1}$ respectively (Figures S15-S16). The UV-Vis spectra from the titration of **2** with H_2PO_4^- show no isosbestic point (Figure 5a and S17) and gave very poor fits when using a 1:1 binding model, while a much better fit was obtained when using a 1:2 binding model (Figure 5a, inset), yielding large K_a values for both the 1:1 and 1:2 host to guest association processes ($1 \cdot 10^6$ and $5 \cdot 10^5 \text{ M}^{-1}$). The formation of a $2 \cdot (\text{H}_2\text{PO}_4^-)_2$ complex is in agreement with the findings from the ^1H NMR titration (Figure 4a) as well as the crystal structure (*vide infra*).

In the case of the titration of **2** with $\text{H}_2\text{P}_2\text{O}_7^{2-}$, an isosbestic point was observed and the data could be fitted to a 1:1 binding model (Figure 5b and S18), giving a $K_{1:1}$ of $6 \cdot 10^6 \text{ M}^{-1}$. During the ^1H NMR titration, the formation of a 2:1 host-guest complex was observed (at 1 mM), but the 200-fold lower concentration of **2** ($5 \mu\text{M}$) would decrease the fraction of this complex present in solution.

As a result, it turns out that macrocycle **2** exhibits the highest affinity of the three macrocycles, regardless of the size of the anion (Table 1). It even outperforms for both Cl^- and H_2PO_4^- , the similarly sized tetraurea macrocycles.⁴⁸ For macrocycle **3** the lower affinity for the different guests can be explained by the need to outcompete the intramolecular hydrogen bonds that can form in this flexible macrocycle, that has furthermore only two aromatic CH protons pointing into the cavity. The difference in affinities between macrocycle **1** and **2** for Cl^- and H_2PO_4^- could be explained by their difference in flexibility. Both

these anions are expected to be too small for macrocycle **1** to optimally exploit all its H-bond donors, whereas macrocycle **2** is most likely able to adapt its conformation so that all four NH H-bond donors can be engaged with each of the anions. More surprising was the fact that macrocycle **2** also outperformed macrocycle **1** in binding $\text{H}_2\text{P}_2\text{O}_7^{2-}$ by three orders of magnitude, since macrocycle **1** was expected to be well preorganized for binding of such larger

guests, based on initial computational studies. As amides were reported to have slightly higher anion binding affinities than acylhydrazones^{34,35} and calculated to be slightly more acidic (see SI Section 1), the difference in binding motifs cannot explain the superior binding by **2**. However, the presence of six CH protons pointing into the cavity of **2** compared to four CH protons for **1** could contribute to its performance.

Table 1. Association constants determined for macrocycles **1**, **2**, and **3** in DMSO.

Host	$K_a \text{ Cl}^-$		$K_a \text{ H}_2\text{PO}_4^-$	$K_a \text{ H}_2\text{P}_2\text{O}_7^{2-}$
	NMR (M^{-1}) ^a	UV-Vis (M^{-1}) ^b	UV-Vis (M^{-1}) ^b	UV-Vis (M^{-1}) ^b
1	$K_{1:1} = (1.2 \pm 0.1) \cdot 10^2$	$K_{1:1} = 78 \pm 4$	$K_{1:1} = (8.9 \pm 1.2) \cdot 10^2$	$K_{1:1} = (3.9 \pm 0.3) \cdot 10^3$
2	$K_{1:1} = (2.1 \pm 0.3) \cdot 10^3$	$K_{1:1} = (1.6 \pm 0.1) \cdot 10^3$	$K_{1:1} = (1.2 \pm 0.8) \cdot 10^6$ $K_{1:2} = (5.0 \pm 0.5) \cdot 10^5$	$K_{1:1} = (6.4 \pm 2.6) \cdot 10^6$
3	$K_{1:1} < 10$	- ^c	- ^c	- ^c

^a) DMSO- d_6 + 0.5% H_2O , 298 K ^b) DMSO, 298 K ^c) not determined. The errors are based on the standard deviations of the results from three titrations.

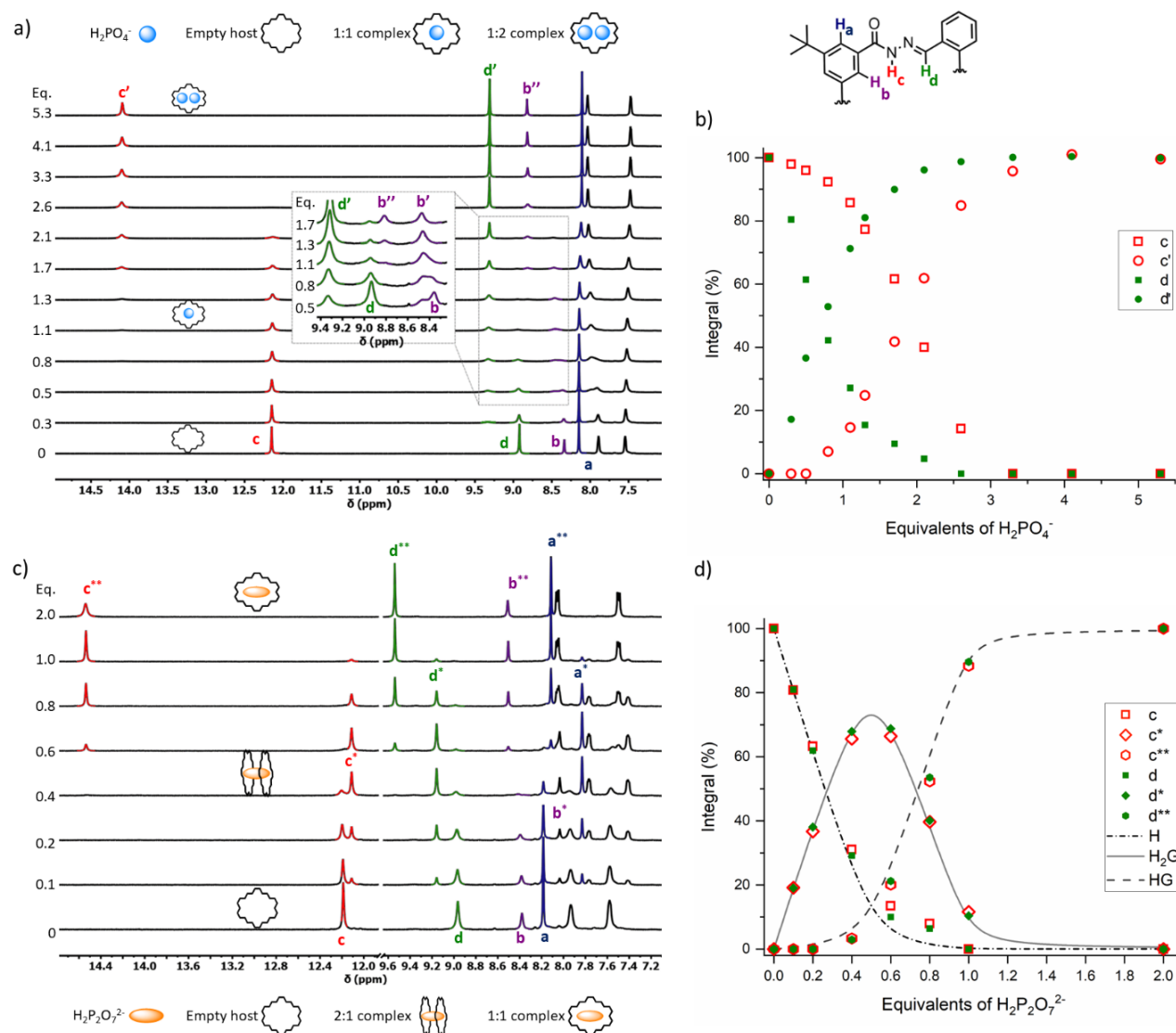


Figure 4. ^1H -NMR titrations of macrocycle **2** (1 mM, DMSO- d_6 + 0.5% H_2O) with TBAH_2PO_4 (a, 600 MHz), and $\text{TBA}_2\text{H}_2\text{P}_2\text{O}_7$ (c, 400 MHz); b) Integrals of signals c, c', d, and d' as % relative to the *tert*-butyl signal over the course of titration with TBAH_2PO_4 ; d) Relative proportions of signals d, d* and d** as well as c, c* and c** throughout the titration with $\text{TBA}_2\text{H}_2\text{P}_2\text{O}_7$ as % of the total integrals of d and c with simulated speciation curves of a 2:1 association (1 mM host, $K_{1:1} = 6.4 \cdot 10^6 \text{ M}^{-1}$, $K_{2:1} = 2 \cdot 10^4 \text{ M}^{-1}$) overlaid to help guide the eye.

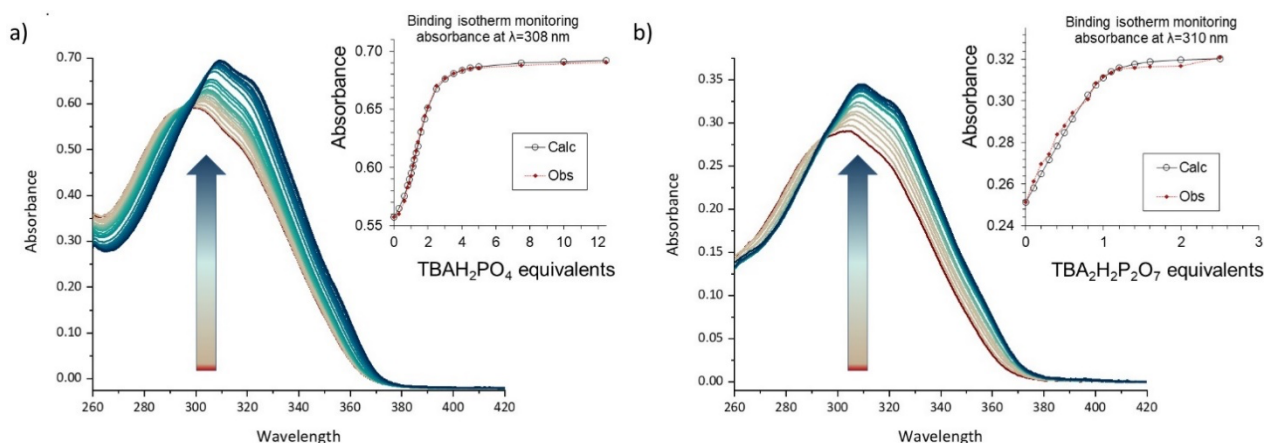


Figure 5. UV-Vis spectroscopy titrations of macrocycle **2** with a) TBAH₂PO₄ (10 μM **2** in DMSO) and b) TBA₂H₂P₂O₇ (5 μM **2** in DMSO).

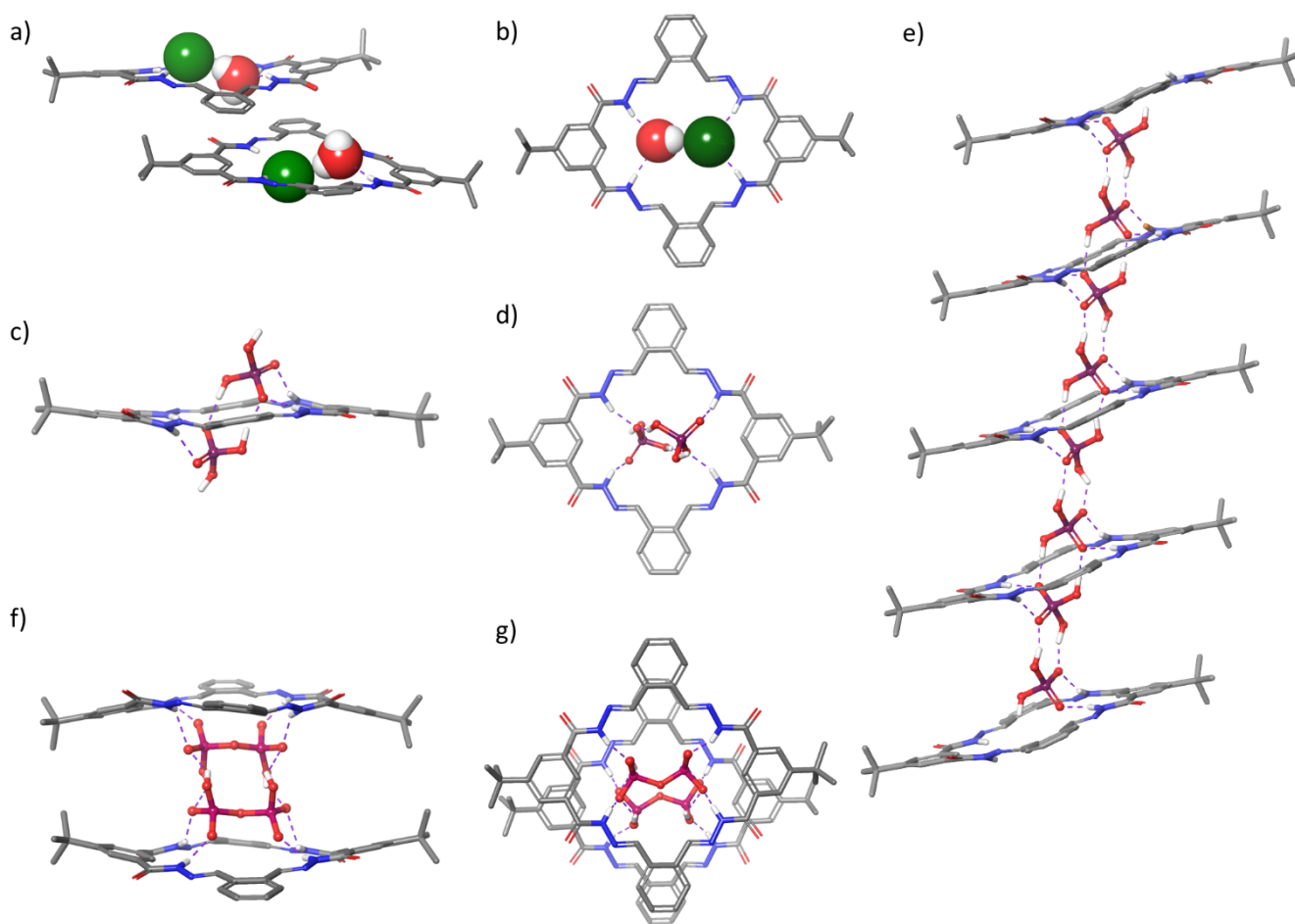


Figure 6. The crystal structures obtained for macrocycle **2** in the presence of Cl⁻, H₂PO₄⁻ and H₂P₂O₇²⁻ in a side view (a, c, f) and a top-down view (b, d, g) as well as a model of the extended structure within the crystal structure of the **2**·H₂P₂O₇²⁻ complex (e). The macrocycle is shown in capped stick representation, H₂PO₄⁻ and H₂P₂O₇²⁻ are shown in ball and stick representation and H₂O and Cl⁻ as space filled representation. Tetrabutylammonium, solvent molecules not found within the binding cavities, as well as non H-bonding hydrogens are omitted for clarity.

X-ray crystallography. To further study the interactions of macrocycle **2** with the different anions, we obtained single crystals of the host-guest systems **2**·Cl⁻, **2**·H₂PO₄⁻, **2**·H₂P₂O₇²⁻, and **2**·malonate and analyzed them by single-crystal X-ray diffraction.

The crystal structure of **2**·Cl⁻ showed in the asymmetric unit the presence of two macrocycles adopting a flat

conformation (Figure 6a). In each macrocycle, two acylhydrazone groups coordinate to one chloride ion, whereas a water molecule fills the remanent space of the cavity (Figure 6b). In each macrocycle, two N-H···Cl hydrogen bonds are observed between two acylhydrazone groups and the anion with N···Cl distances in the range of 3.325 Å to 3.382 Å. In addition, the water molecule

interacts with the remaining acylhydrazone group through the formation of two N-H...O_w hydrogen bonds with N...O_w distances in the range of 3.007 Å to 3.328 Å, supporting the flat conformation of the macrocycle. The presence of both guest molecules in each macrocycle cavity is stabilized by one O_w-H...Cl interaction with O_w...Cl distances of 3.145 Å and 3.191 Å.

In the complex **2**·H₂PO₄⁻, the macrocycle shows a flat conformation similar to the previous one for **2**·Cl⁻. However, in this case, two molecules of H₂PO₄⁻ anions are observed in the macrocycle cavity (Figure 6c-d), which corroborates the 1:2 host-guest stoichiometry observed in the binding studies by NMR and UV-Vis titration experiments (Figures 4a and 5a). This association is favored by the formation of two different N-H...O hydrogen bonds with N...O distances of 2.728 Å to 2.785 Å between the anion with the four acylhydrazone groups of the macrocycle. Interestingly, the hydrogen atoms in the H₂PO₄⁻ anion allow the formation of O-H...O interactions, with O...O distances of 2.525 Å, between the anions producing a linear polymeric chain running at an angle of approximately 121° to the plane of the macrocycles (Figure 6e).^{54,55}

Finally, for the **2**·H₂P₂O₇²⁻ complex, a 1:1 ratio between the macrocycle and the anion is observed, favored by the size of the H₂P₂O₇²⁻ anion, allowing its interaction with all the four acylhydrazone groups. The four different N-H...O hydrogen bonds have a N...O distance in the range of 2.683 Å to 2.825 Å. Once again, the hydrogen atoms in the H₂P₂O₇²⁻ anion allow interactions between anions. Nevertheless, this association was observed for only two anions, forming a dimer rather than a linear network as observed for H₂PO₄⁻. The crystal lattice of **2**·H₂P₂O₇²⁻ can be described as a sandwich-type complex in which two macrocycle molecules surround two anions (Figure 6f-g).⁵⁶⁻⁵⁸

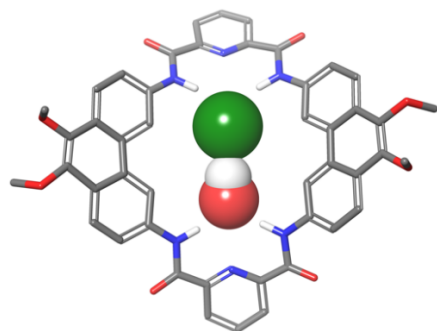


Figure 7. The crystal structure obtained for macrocycle **1** in the presence of Cl⁻. The macrocycle is shown in capped stick representation and H₂O and Cl⁻ as space filled representation. Hexyl side chains, Tetrabutylammonium, solvent molecules not found within the binding cavities as well as non H-bonding hydrogens are omitted for clarity.

The crystal structure of a complex of **1**·Cl⁻ was also obtained, showing a chloride and a water molecule bound within the cavity of the macrocycle (Figure 7), similar as for the complex of **2**·Cl⁻. In this case however, the macrocycle forms a sandwich complex of two macrocycles, two chloride ions and two water molecules. The macrocycle adopts a saddle conformation to allow it to maintain N...Cl distances between the Cl⁻ and the amide N-H in the range of 3.22-3.43 Å and N...O_w distances between the water

molecule and the amide N-H in the range of 2.93-3.09 Å. The complex is further stabilized by the interaction of the two guest molecules with each other with O_w...Cl distances of 3.19 Å and 3.21 Å as was the case for **2**·Cl⁻.

Conclusion

Three new macrocycles with a 26-membered interior ring size and four NH-based binding moieties have been prepared. The three macrocycles were shown, both by computational modelling as well as 1D-ROESY experiments, to exhibit significantly different degrees of preorganization, originating from different degrees of rigidity within their macrocyclic structures. Binding studies with anions of varying size (Cl⁻, H₂PO₄⁻, and H₂P₂O₇²⁻) revealed very significant differences between the macrocycles. The highly flexible macrocycle **3** favors intramolecular hydrogen bonding over interactions with anions. The rigid macrocycle **1** exhibits, not unexpectedly, poor interaction with Cl⁻ since the cavity was too large, but showed good interactions with H₂PO₄⁻ and H₂P₂O₇²⁻. Macrocycle **2** exhibits the best anion affinity for all three anions, most likely due to the combination of its preorganization and its ability to adapt its conformation more easily than macrocyclic receptor **1**. Furthermore, in addition to four acylhydrazone NH H-bond donors, it has six aromatic protons pointing into the binding cavity. X-ray crystallography of complexes of macrocycle **2** with the three anions confirmed its ability to bind these different anions, of which H₂PO₄⁻ was bound with a 1:2 host to guest stoichiometry.

These three macrocycles of the same size but varying preorganization and flexibility show that the macrocycle featuring an intermediate flexibility provides the best binding affinity for a range of anions of different size and shape. While several studies reported that more rigidity of preorganized macrocyclic structure provides better affinities, our study illustrates that some flexibility in the macrocycle can be beneficial, in particular for the binding of anions with more complex geometries. This insight will help to guide the design of future generations of macrocyclic receptors for anions or other guests.

Experimental Section.

Synthesis and Characterization. Commercially available solvents and chemical reagents were used as received unless otherwise stated. Purification by Flash Column Chromatography (FCC) was carried out using silica gel with a 60 Å pore size and a particle size of 40-63. Unless otherwise stated, glassware employed for moisture sensitive reactions were flame dried prior to use. NMR spectra were acquired using a Varian VNMRs 400 spectrometer or a JEOL JNM-ECZ400R/S3 operating at 9.4 T (400 MHz for ¹H) equipped with a double resonance OneNMR™ or ROYAL™ probe respectively or a JEOL JNM-ECZ600R/S3 spectrometer operating at 14.1 T (600 MHz for ¹H) equipped with a double resonance ROYAL™ probe. The ¹H spectra were recorded at 298 K unless otherwise stated and, for titration experiments, using a spectral width of 20.0 or 24.0 ppm centered at 5.0 or 6.0 ppm, 3.0 or 4.0 s relaxation delay, 2.43 or 2.6 μs high-power RF pulse width (flip angle of 30°) and 2.2 or 3.0 s acquisition time. Processing was performed using MestReNova

14.3.1-31739. It comprised zero-filling (total of 64k or 256k points), single exponential or cosine square multiplication of the free induction decay, Fourier transform, phase correction, baseline correction, and chemical shift referencing of the spectrum. The ^1H spectra were referenced with respect to the residual signal of the solvent (methanol- d_3 : 3.31 ppm; acetone- d_5 : 2.05 ppm, DMSO- d_5 : 2.50 ppm) and chemical shifts are reported in ppm. Melting points were recorded on a Stuart SMP20 melting points apparatus and are uncorrected. Mass spectrometry was carried out using a QTOF-6520 Agilent instrument.

9,10-bis(hexyloxy)phenanthrene-3,6-diamine dihydrochloride (7·2(HCl)). 3,6-dibromo-9,10-dihexoxyphenanthrene **5** (100 mg, 186 μmol), Pd(dba) $_2$ (10.72 mg, 18.65 μmol) and Xphos (35.84 mg, 372 μmol) was mixed in a 5 mL flask and placed under an argon atmosphere. Dry toluene (1 mL) was added and the resulting mixture heated to 80°C on a heating mantle followed by addition of benzophenone amine (62.5 μL , 372 μmol). After stirring the solution at 80°C overnight, it was filtered over celite followed by concentration and purification by FCC using 10% EtOAc/Hep as the eluent to yield the crude diimine as a yellow sticky solid. This solid was subsequently dissolved in 1 mL of THF followed by addition of 1 mL of concentrated HCl (37% in H $_2$ O). The resulting precipitate was filtered off on a sintered glass funnel, washed thoroughly with THF (4x 2 mL) and dried in high vacuum to obtain the desired diamine as a white powdery HCl salt (92 mg, 71% over two steps). Decomposition > 210°C. ^1H NMR (400 MHz, CD $_3$ OD) δ 8.80 (d, J = 2.1 Hz, 2H), 8.44 (d, J = 8.8 Hz, 2H), 7.73 (dd, J = 8.8, 2.0 Hz, 2H), 4.26 (t, J = 6.6 Hz, 4H), 1.99 – 1.87 (m, 4H), 1.67 – 1.54 (m, 4H), 1.46 – 1.35 (m, 8H), 0.99 – 0.89 (m, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, CD $_3$ OD) δ 144.9, 131.6, 130.3, 129.7, 126.1, 123.6, 118.8, 75.0, 32.8, 31.4, 27.1, 23.7, 14.4. HRMS ($\text{M}+\text{H}^+$): calc. for C $_{26}\text{H}_{37}\text{N}_2\text{O}_2^+$: 409.2850, found: 409.2858.

Macrocycle 1. To a solution of tetrabutylammonium chloride (2.31 g, 8.31 mmol) in dry DCM (160 mL) under an argon atmosphere was added a solution of pyridine-2,6-dicarbonyl dichloride **8** (41.13 mg, 187 μmol) in dry DCM (20 mL) and a solution containing a mixture of **7·2(HCl)** (90 mg, 187 μmol) and DIPEA (121 mg, 934 μmol) in dry DCM (20 mL) dropwise over the course of 10 hours. After complete addition, the mixture was allowed to stir for an additional 8 hours followed by concentration of the reaction mixture onto celite for purification by FCC, first using 25% acetonitrile/toluene as the eluent to yield the crude macrocycle. A second column using 1% MeOH/DCM as the eluent was employed to obtain the pure macrocycle as an off-white powder (27 mg, 27 %). Mp: > 300°C. ^1H NMR (400 MHz, acetone- d_6) δ 10.78 (s, 4H), 8.48 (d, J = 7.8 Hz, 4H), 8.45 (d, J = 1.9 Hz, 4H), 8.34 (dd, J = 8.9, 1.8 Hz, 4H), 8.32 – 8.25 (m, 6H), 4.21 (t, J = 6.6 Hz, 8H), 1.97 – 1.88 (m, 8H), 1.65 – 1.55 (m, 8H), 1.46 – 1.37 (m, 16H), 0.97 – 0.91 (m, 12H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, acetone- d_6) δ 162.2, 149.4, 142.7, 139.5, 135.9, 128.5, 127.1, 125.5, 123.7, 122.7, 115.4, 73.4, 31.7, 30.4, 25.9, 22.5, 13.5. HRMS ($\text{M}+\text{K}^+$): calc. for C $_{66}\text{H}_{74}\text{N}_6\text{O}_8\text{K}^+$: 1117.5200, found: 1117.5164.

Macrocycle 2. Dihydrazide **10** (500 mg, 2 mmol) was suspended in ethanol (100 mL) and heated with a heat gun until a slightly hazy solution was obtained. This solution was then allowed to cool to 35°C and added in one portion to a vigorously stirred mixture of

phthalaldehyde (267 mg, 2 mmol) and tetrabutylammonium dihydrogenphosphate (339 mg, 1 mmol) in ethanol (100 mL). After stirring overnight a precipitate had formed which was filtered off on a sintered glass funnel, washed thoroughly with ethanol (8x 15 mL) and dried in high vacuum to yield the desired macrocycle as a white powder (397 mg, 57 %). Mp: Decomposition > 230°C. ^1H NMR (400 MHz, DMSO- d_6) δ 12.19 (s, 4H), 8.97 (s, 4H), 8.38 (t, J = 1.5 Hz, 2H), 8.19 (d, J = 1.5 Hz, 4H), 7.93 (dd, J = 5.8, 3.4 Hz, 4H), 7.58 (dd, J = 5.9, 3.3 Hz, 4H), 1.41 (s, 18H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, DMSO- d_6 , +3 eq TBAH $_2$ PO $_4$) δ 163.3, 151.1, 144.6, 133.7, 133.7, 129.6, 128.6, 125.7, 123.1, 34.6, 31.0. HRMS ($\text{M}+\text{H}^+$): calc. for C $_{40}\text{H}_{41}\text{N}_8\text{O}_4^+$: 697.3245, found: 697.3216.

2,2'-(((4,5-diethoxy-1,2-phenylene)bis(methylene))bis(oxy))bis(isoindoline-1,3-dione) (15). To a stirred solution of N-hydroxyphthalamide (926 mg, 5.68 mmol) and 1,2-bis(bromomethyl)-4,5-diethoxybenzene **13** (1 g, 2.8 mmol) in dry DMF (15 mL) under argon was added DBU (1 mL, 6.8 mmol) over 5 minutes. Following complete addition, the mixture was left to stir for 25 minutes after which HCl (1N aq. solution, 20 mL) was added and the resulting precipitate filtered on a sintered glass funnel followed by washing with water (3x 10 mL). The wet solid was suspended in toluene for azeotropic removal of water twice over after which the resulting solid was dried in high vacuum to obtain the desired compound as a white solid (1.4 g, 95 %). Mp: Decomposition > 220°C. ^1H NMR (400 MHz, DMSO- d_6) δ 7.88 – 7.80 (m, 8H), 7.12 (s, 2H), 5.41 (s, 4H), 4.01 (q, J = 7.0 Hz, 4H), 1.27 (t, J = 7.0 Hz, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, DMSO- d_6) δ 163.2, 148.3, 134.8, 128.5, 126.5, 123.2, 116.3, 76.3, 63.8, 14.5. HRMS ($\text{M}+\text{H}^+$): calc. for C $_{28}\text{H}_{25}\text{N}_2\text{O}_8^+$: 517.1605, found: 517.1591.

O,O'-(((4,5-diethoxy-1,2-phenylene)bis(methylene))bis(hydroxylamine) (16). To a solution of protected hydroxylamine **15** (400 mg, 774 μmol) in ethanol (5 mL) was added hydrazine hydrate (85 μL , 1.7 mmol) and the resulting mixture stirred overnight at room temperature. The mixture was evaporated onto celite for purification by FCC using 5% MeOH/DCM as the eluent to yield the desired bis(hydroxylamine) as a white solid (180 mg, 90%) Mp: 104-106°C. ^1H NMR (400 MHz, DMSO- d_6) δ 6.89 (s, 2H), 5.97 (s, 2H), 4.55 (s, 4H), 4.01 (q, J = 7.0 Hz, 4H), 1.31 (t, J = 7.0 Hz, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, DMSO- d_6) δ 147.2, 128.9, 114.6, 74.2, 63.8, 14.8. HRMS ($\text{M}+\text{H}^+$): calc. for C $_{12}\text{H}_{21}\text{N}_2\text{O}_4^+$: 257.1496, found: 257.1484.

Macrocycle 3. To a stirred solution of tetrabutyl ammonium chloride (108 mg, 390 μmol) and triethylamine (20 μL , 156 μmol) in dry DCM (5 mL) under an argon atmosphere was added solutions of 5-(*tert*-butyl)isophthaloyl dichloride **17** (10.11 mg, 5 mM in dry DCM) and bis(hydroxylamine) **16** (10 mg, 5 mM in dry DCM) dropwise over the course of 30 minutes. The solution was left to stir overnight after which it was evaporated onto celite for purification by FCC using 25 % acetonitrile/toluene to yield the desired macrocycle as a white solid (9.5 mg, 55 %). Mp: Decomposition > 260°C. ^1H NMR (400 MHz, DMSO- d_6) δ 12.11 (s, 4H), 8.24 (t, J = 1.6 Hz, 2H), 8.06 (d, J = 1.6 Hz, 4H), 6.97 (s, 4H), 5.07 (s, 8H), 3.92 (q, J = 7.0 Hz, 8H), 1.37 (s, 18H), 1.21 (t, J = 7.0 Hz, 12H). $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, DMSO- d_6) δ 163.7, 151.3, 147.7, 132.0, 127.9, 127.3, 123.4, 116.8, 75.1, 63.8,

34.8, 30.9, 14.6. HRMS (M+H⁺): calc. for C₄₈H₆₁N₄O₁₂⁺: 885.4280, found: 885.4279.

General procedures for ¹H-NMR titrations. All titrations were carried out at 1 mM macrocycle concentration in DMSO-*d*₆ with 0.5% H₂O. Stock solutions of guests were prepared using the host solution to ensure that no dilution of the host takes place over the course of the titration. TBA salts were dried under high vacuum prior to the preparation of stock solutions with concentrations in the range of 20–200 mM depending on the titration. Aliquots of guest stock solution were added sequentially to an NMR tube with the free host and a proton spectrum acquired for each addition. The chemical shift of NH protons and other relevant signals were fitted to a 1:1 binding model using Bindfit.^{44–46}

General procedures for UV-Vis titrations. Titrations were carried out at concentration ranges of 5–15 μM in DMSO. Stock solutions of guests were prepared using the host solution to ensure that no dilution of the host takes place over the course of the titration. TBA salts were dried under high vacuum prior to the preparation of stock solutions with concentrations in the range of 0.5–100 mM. Aliquots of guest stock solution were added sequentially to a cuvette with the free host and a UV-Vis absorption spectrum acquired for each addition on a Shimadzu UV-3600 UV-Vis spectrometer at 25°C. The change of absorption at relevant wavelengths were fitted to an appropriate binding model (1:1 or 1:2 host to guest models) using Bindfit (Nelder-Mead fit method).^{44–46}

Molecular modelling. Initial modelling was done by Monte Carlo multiple minimum (MCMM) conformational searches (100 steps per torsion angle, maximum 1000 steps in total), performed in Schrödinger Release 2019-1, using the OPL3e force field without implicit solvation in Maestro MacroModel (version 11.9.011). The structure obtained after this first run of Molecular Mechanics geometry optimization runs were further minimized using the semi-empirical MOPAC2016 package (Version: 22.110W, James J. P. Stewart) using the PM7 Hamiltonian (full coordinates geometry optimization, MM correction to CONH barrier, convergence for gradient norm below 0,10).

X-ray crystallography. Generally, single crystals were grown from 1–4 ml solutions of macrocycle (1–3 mM). Single crystals of **2**·Cl[−] were grown by slow evaporation of a dioxane solution of **2** in the presence of excess TBACl. Single crystals of **2**·H₂PO₄[−] were obtained by slow evaporation of a DMSO/acetone solution of **2** in the presence of excess TBAH₂PO₄. Single crystals of **2**·H₂P₂O₇^{2−} were obtained by slow evaporation of a chloroform solution of **2** in the presence of excess TBA₂H₂P₂O₇. Single crystals of **2** with malonate were obtained by slow evaporation of a dioxane solution of **2** in the presence of excess TBA₂C₃H₂O₄. Single crystals of **1**·Cl[−] were grown by slow evaporation of a chloroform solution of **1** in the presence of excess TBACl. Single crystals of **3** were obtained by slow evaporation of a dioxane solution of **3**.

Diffraction data were collected using the Oxford Diffraction Gemini R Ultra diffractometer (Cu Kα, multilayer mirror, Ruby CCD area detector) at 100(2) K except **2**·H₂PO₄[−] was collected at 295(2) K. Data collection, unit cells determination and data reduction were carried out using CrysAlis PRO software package⁵⁹ using Olex2⁶⁰ and shelXle⁶¹, the structure was solved with the SHELXT 2015⁶²

structure solution program by Intrinsic Phasing methods and refined by full-matrix least squares on |F|² using SHELXL-2018/3.⁶⁰ Non-hydrogen atoms were refined anisotropically, while C–H hydrogen atoms were placed in geometrically calculated positions using a riding model. Additional details of the refinement are given in the SI. CCDC deposition numbers are 2311610–2311615.

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