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A Tris-Macrocycle with Proton Sponge Characteristics as Efficient Receptor for Inorganic Phosphate and Nucleotide Anions

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Synthesis, characterisation and proton-sponge behaviour of a new linear constrained tris-macrocycle (**L**), containing three cyclen (cyclen = 1,4,7,10-tetraazacyclododecane) reinforced macrocycles connected by two 2,6-pyridinediylbis(methylene) linkers, is reported. Protonated forms of **L** are efficient receptors for inorganic phosphate and nucleotide anions (ATP and ADP). The binding properties of **L** toward these substrates have been investigated in aqueous solution by means of potentiometric titrations, determining the stability constants of the adducts. The interaction mode was clarified by using ^{1}H and ^{31}P NMR measurements in aqueous solution and by means of molecular modelling calculations carried out on differently protonated species of **L** as well as on several nucleotide-receptor adducts. The stability of the adducts is mainly due to charge-charge and hydrogen bonding interactions between the polyphosphate chain of nucleotides and the ammonium groups of **L**.

Introduction

Anion binding, the coordination of chemical species by virtue of their anionic nature, plays a central role in biological processes.[1] For instance, a large variety of substrates and cofactors engaged in biological processes are anions. From this point of view, inorganic phosphates and nucleotides are ubiquitously present in biological systems and play crucial roles in many cellular functions, such as transport across membranes, DNA synthesis, cell signalling and energy or electron-transfer processes.[1] In most cases, these functions are regulated by recognition processes involving proteins able to selectively bind the appropriate phosphate or nucleotide anion by using essentially non covalent interactions, such as charge–charge and charge–dipole interactions, hydrogen bonding and hydrophobic effects.

In this context, the design of synthetic receptors able to bind phosphate anions in aqueous solution has represented and still represents one of the approaches to the analysis of the weak forces which regulate the recognition processes in biological systems.[2] Actually, several examples of phosphate anion binding by synthetic receptors, mostly of polyammonium type, have been recently reported.[3–18] It is now accepted that the formation of stable host-guest adducts requires the incorporation of sites for multiple interactions with substrates. In particular, to achieve a better recognition of nucleotide anions, the receptor needs to contain not only positively charged ammonium groups able to interact with the anionic phosphate moiety, but also binding sites able to interact via hydrogen bonding or π-stacking with the nucleobases. Finally, the binding sites of the receptor need to be suitably preorganised within the receptor structure to optimally interact with the anionic substrate. Although many examples of polyammonium ligands with different molecular structures are known, less attention has been devoted to "structurally reinforced" polyamine receptors containing amine groups linked together by two short aliphatic chains. On the other hand, structural modifications of tetraaza macrocycles based on the ethylene bridging of adjacent[19] (side-bridged) or opposite[20] (cross-bridged) nitrogen atoms have been recently reported according to various procedures. The resulting bridged polyazacycloalkanes have been shown to exhibit unusual properties in metal coordination[21] and also to act as strong bases in aqueous solution.[22] We have previously also shown that the binding properties toward metal cations displayed by these reinforced tetraamine ligands are persisting also in their polycyclic derivatives.[23]

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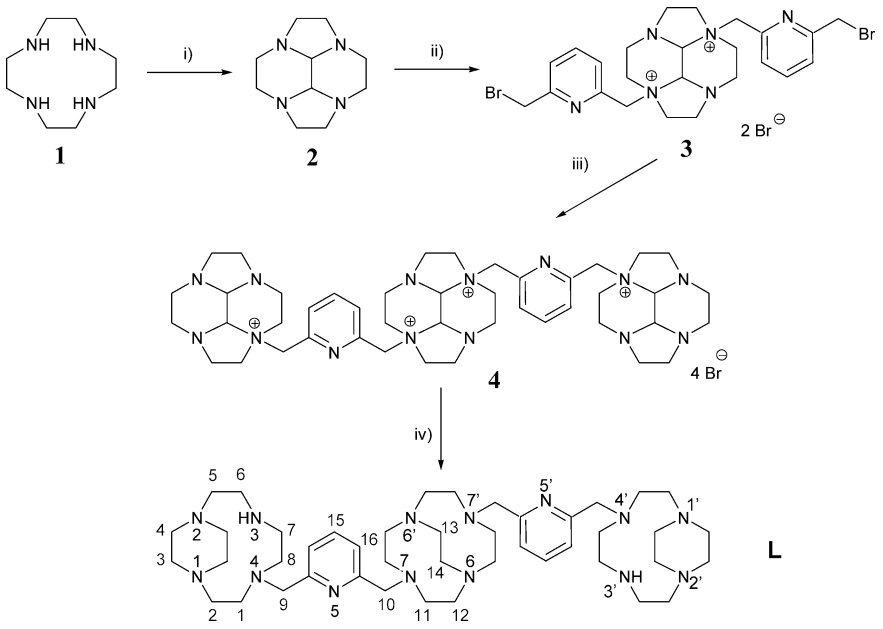


Figure 1. Synthesis of **L** and ligand drawing with atom labelling used in NMR experiments. i) MeOH/glyoxal, ii) 2,6-bis(bromomethyl)-pyridine/CH_3CN, iii) **2**/DMF, iv) $NaBH_4$/EtOH.

We have now synthesized a new molecule (**L**) containing three 1,4,7,10-tetraazacyclododecane (cyclen) reinforced rings connected by two 2,6-pyridinediylbis(methylene) linkers (Figure 1). This ligand contains both side-bridged and cross-bridged amine groups and is obtained in a four steps procedure starting from cyclen using bis-aminal intermediates for the macrocycle selective alkylation[24] and for the formation of the ethylene bridges.[19,20] In principle, this ligand may behave as multifunctional receptor for anionic forms of nucleotides, through the formation of salt bridges between the ammonium groups and the phosphate chains and π-stacking and hydrophobic interactions between the pyridine unit and the adenine moiety of substrates.[17]

In this paper we report the results of a potentiometric, ^1H and ^{31}P NMR and molecular modelling study on the interaction of phosphate, diphosphate, triphosphate, ATP and ADP with variously protonated form of **L**, in order to elucidate the correlation between the stability of the supramolecular adducts formed and the structure of the receptor.

Results and Discussion

Synthesis of the Ligand L

The macrocyclic bis-aminal **2** is cleanly obtained from the condensation of glyoxal with cyclen **1** as described previously.[25] Reaction of one equivalent of a biselectrophile with **2** (see Figure 1) leads to an N^1,N^7-dialkylation of the macrocycle via a diquat salt. The diammonium salt **3** was then allowed to react with an excess of cyclen-glyoxal **2** giving the tetra-salt **4**. The latter yielded the linear constrained tris-macrocycle **L** after filtration and cleavage of the bis-aminal bridges with $NaBH_4$ in absolute ethanol.

Basicity Properties of the Receptor

A necessary preliminary requisite for the investigation of the binding properties of the receptor in its protonated forms is the knowledge of its basicity properties. The protonation equilibria of **L** have been studied first by means of potentiometric measurements in aqueous solution at 298.1 ± 0.1 and 313.1 ± 0.1 K in 0.1 mol dm^{-3} NMe_4Cl. **L** behaves as a very strong base in the first and in the second protonation step; in fact, deprotonation of the diprotonated species H_2L^{2+} is not detected by means of potentiometric titrations, at least in the pH range investigated (2–12). Furthermore, the ^1H NMR spectra of the ligand in 0.5 M NaOH is equal to that recorded at pH 11.5, indicating that the H_2L^{2+} species cannot be deprotonated even in strongly alkaline solution, i.e., receptor **L** behaves as a "biproton sponge".[26] Actually, H_2L^{2+} gives rise, in the pH range 2–12, to six protonation equilibria, which are listed in Table 1 together with the corresponding protonation constants. The distribution diagram of the protonated species at 313.1 K is shown in Figure 2, while that at 298.1 K is reported in the Supporting Information (Figure S1).

Pyridine nitrogen atoms are characterized by far lower basicity than aliphatic amine N atoms, the protonation constant of pyridine being 5.67 log units,[27] while the first protonation constant of secondary amines usually ranges between 9 and 11 logarithmic units. Therefore, it is expected that at least the first two protonation steps of H_2L^{2+} take place on the amine groups of the aliphatic chains. To support this hypothesis and to determine the localisation of the acidic protons in all the protonated specie of the receptor, we performed ^1H NMR measurements in aqueous solutions at different pH values. The spectra present a marked fluxionality at room temperature and for this reason the ^1H

Table 1. Protonation constants of **L**, determined in 0.1 mol dm^{-3} NMe_4Cl at 298.1 ± 0.1 K and 313.1 ± 0.1 K (values in parentheses are standard deviation in the last significant digit).

	log *K*	
	T = 298.1 K	*T* = 313.1 K
$H_2L^{2+} + H^+ = H_3L^{3+}$	10.6(1)	9.7(1)
$H_3L^{3+} + H^+ = H_4L^{4+}$	7.78(5)	7.0(1)
$H_4L^{4+} + H^+ = H_5L^{5+}$	5.49(5)	5.1(1)
$H_5L^{5+} + H^+ = H_6L^{6+}$	4.67(5)	4.3(1)
$H_6L^{6+} + H^+ = H_7L^{7+}$	3.22(6)	2.9(1)
$H_7L^{7+} + H^+ = H_8L^{8+}$	2.2(1)	2.0(1)

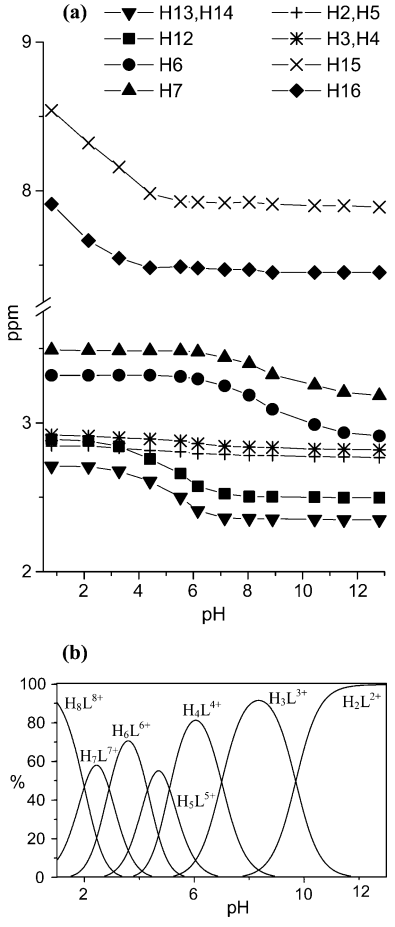


Figure 2. (a) pH dependence of selected ^{1}H NMR signals of the ligand (313.1 K, I = 0.1 M), (b) distribution diagram of the protonated species of the ligand.

NMR study was performed at 313.1 K. The pH dependence of selected ^{1}H NMR signals of the receptor is reported in Figure 2 (the chemical shifts of the signals of protons H1, H8 and H11 do not show significant changes in the pH range investigated and they are omitted).

At pH 12.0 the spectrum exhibits eleven signals, nine for the aliphatic protons and two for the aromatic ones. In particular, the aliphatic portion of the spectrum is featured by seven multiplets at δ = 2.21 ppm (attributed to the hydrogen atoms H1, H8 and H11), 2.77 ppm (H2 and H5), 2.82 ppm (H3 and H4), 2.93 ppm (H6), 3.21 ppm (H7), 2.44 ppm (H12), 2.30 ppm (H13 and H14), and by two singlets at δ = 3.93 ppm for H9 and 4.12 ppm for H10 (see Figure 1 for atom labelling), while the aromatic region of the spectrum displays two multiplets for the protons H15 and H16 at δ = 7.89 ppm and 7.45 ppm, respectively. All the assignments have been made on the basis of ^{1}H-^{1}H homonuclear correlation experiments at the different pH values studied. The spectral features account for a time averaged C_{2v} symmetry of the molecule, which is preserved throughout all the pH range investigated.

The analysis of the ^{1}H spectrum of the receptor may suggest a tentative localisation of the two acidic protons in the $H_2\mathbf{L}^{2+}$ species. In fact, the methylene groups H2, H3, H4 and H5, adjacent to N1 and N2 give rise to two multiplets at δ = 2.77 ppm (for H2 and H5) and 2.82 ppm (for H3 and H4). These chemical shifts are unusually high for a CH_2 group adjacent to a tertiary nitrogen atom. For instance the CH_2 groups H12, H13 and H14 give two signals at δ = 2.44 ppm and 2.30 ppm. Furthermore, the signals of H2, H3, H4 and H5 do not vary significantly in all the pH range investigated. These observations suggest that in $H_2\mathbf{L}^{2+}$ the two acidic protons are shared between N1 and N2 and between the symmetry related N1′ and N2′ nitrogen atoms, adjacent to the methylene groups H2, H3, H4 and H5. Therefore, each lateral reinforced macrocycle shows a proton sponge behaviour. At a first glance, the fact that the central reinforced tetrammine ring does not display "proton sponge" characteristics can be somewhat surprising. On the other hand, the increased positive charge gathered on the receptor with the formation of the $H_2\mathbf{L}^{2+}$ species reduces the proton affinity of the central macrocyclic ring. Actually, a similar behaviour has been found in a bis-macrocyclic receptor composed by two reinforced tetraamine rings separated by a benzene unit.[23b]

In the pH-range 11.5–6.0 where the first two protons bind to the ligand, the signals of the hydrogen atoms H6 and H7, in the α-position with respect to N3, exhibit a downfield shift, whereas the other signals do not shift appreciably, suggesting that in the $H_4\mathbf{L}^{4+}$ species the two acidic protons are localized on the secondary nitrogens N3 and N3′ of the lateral macrocyclic units. Finally, in the pH range 5.5–3.5 the receptor binds two further protons, giving the $H_5\mathbf{L}^{5+}$ and $H_6\mathbf{L}^{6+}$ species. The formation of this hexaprotonated form is accompanied by a downfield shift of the H12, H13 and H14 protons (see Figure 2). These spectral features indicate that the fifth and sixth protonation steps take place on the N6 and N6′ nitrogens of the central tetraamine ring.

It is to be noted that the signals of the aromatic protons H15 and H16 do not display significant shifts in the pH range 11.5–4.0, indicating that the heteroaromatic nitrogens are not involved in proton binding. Conversely, a downfield shift is observed for the resonances of H15 and H16 only below pH 4, where the hepta- and octa-protonated species are formed in solution. This may suggest that in the $H_7\mathbf{L}^{7+}$ and $H_8\mathbf{L}^{8+}$ species the two acidic protons are localized on the pyridine N atoms N5 and N5′.

Phosphate Anion Binding in Aqueous Solution

Binding of phosphate, diphoshate, triphosphate, ATP and ADP by $H_2\mathbf{L}^{2+}$ has been studied by means of potentiometric and ^1H and ^{31}P NMR measurements in aqueous solution. In fact, protonation of **L** enables the receptor to form stable adducts with all the anionic species of substrates investigated. Analysis of the potentiometric data with the program HYPERQUAD[28] allows one to determine the species formed in solution and their overall formation constants β_{HLA} (A = PO_4^{3-}, $P_2O_7^{4-}$, $P_3O_{10}^{5-}$, ADP^{3-}, ATP^{4-}), relative to equilibria of the type $H_2L + A + nH = [H_{n+2}LA]$, which are listed in Tables 2 and 3. Figure 3 displays the distribution diagrams of the adducts with ATP and ADP, while those with the inorganic phosphate anions are given within the Supporting Information (Figure S2). The data in Tables 2 and 3 and in Figure 3 support the assumption of the formation of a large number of 1:1 adducts with different protonation degree. Substrate complexation occurs in a wide pH range (for instance ATP and ADP are almost completely bound by the polyammonium receptor between pH 3 and 9).

Table 2. Overall stability constants (log β) of the PO_4^{3-}, $P_2O_7^{4-}$ and $P_3O_{10}^{5-}$ adducts with $H_2\mathbf{L}^{2+}$, determined by means of potentiometric measurements in 0.1 mol dm^{-3} NMe_4Cl at 298.1 ± 0.1 K (charges omitted for clarity, value in parentheses are standard deviation in the last significant digit).

	log β		
	PO_4^{3-}	$P_2O_7^{4-}$	$P_3O_{10}^{5-}$
$H_2L + A + H = H_3LA$	14.99 (3)		
$H_2L + A + 2H = H_4LA$	26.28 (3)	23.44 (4)	
$H_2L + A + 3H = H_5LA$	34.13 (3)	31.92 (3)	32.19 (8)
$H_2L + A + 4H = H_6LA$	41.44 (2)	38.13 (4)	38.20 (9)
$H_2L + A + 5H = H_7LA$	46.99 (2)	44.43 (5)	44.40 (8)
$H_2L + A + 6H = H_8LA$	51.76 (3)	52.77 (5)	49.41 (8)
$H_2L + A + 7H = H_9LA$	55.08 (4)	53.38 (5)	53.56 (5)

Table 3. Overall stability constants (log β) of the ATP^{4-} and ADP^{3-} adducts with $H_2\mathbf{L}^{2+}$, determined by means of potentiometric measurements in 0.1 mol dm^{-3} NMe_4Cl at 298.1 ± 0.1 K (charges omitted for clarity, value in parentheses are standard deviation in the last significant digit).

	log β	
	ADP^{3-}	ATP^{4-}
$H_2L + A + H = H_3LA$	14.89(1)	
$H_2L + A + 2H = H_4LA$	23.37(1)	23.22 (6)
$H_2L + A + 3H = H_5LA$	29.87(1)	32.14(9)
$H_2L + A + 4H = H_6LA$	35.58(1)	38.24(8)
$H_2L + A + 5H = H_7LA$	40.48(1)	42.95(8)
$H_2L + A + 6H = H_8LA$	43.81(1)	47.86(9)
$H_2L + A + 7H = H_9LA$		51.87(8)

Although the formation of both 1:1 and 2:1 anion/receptor adducts has been observed in some previously reported cases,[29] the data analysis under our experimental conditions reveals only 1:1 stoichiometries for all species detected in our systems. The determination of the stepwise formation constants of the adducts between the protonated receptor and the anionic substrates, i.e., the constants relative to equilibria of the type $H_{n-i}L + H_iA = [H_nLA]$, implies the knowledge of the localisation of the acidic protons on receptor and substrates. This task is made difficult in the present case by the presence in aqueous solution of a large number of overlapping equilibria in the same pH range and by the values of the protonation constants of the substrates, which are quite similar, in some cases, to the protonation constants of the receptor. In these cases, in fact, two different equilibria can be proposed. For instance, the H_9LATP adduct could be formed either through the equilibria $H_6L + H_3ATP = H_9LATP$ or $H_7L + H_2ATP = H_9LATP$, with equilibrium constants of 7.22 and 7.28 respectively. The values of the stability constants of the adducts, calculated by using this approach are given within the Supporting Information (Tables S3 and S4). To overcome the presence of different equilibria present in solution at the same pH value, it can be useful to calculate effective stability constants.[30] For a given pH value, if the total amount of free substrate ($\Sigma H_{(h-i)}A$), free receptor [$H_{(i)}L$] and adduct formed (H_hLA) are known, one can define an effective stability constant by using the equation $K_{eff} = \Sigma H_hLA/[\Sigma H_{(h-i)}A\ \Sigma H_iL]$.

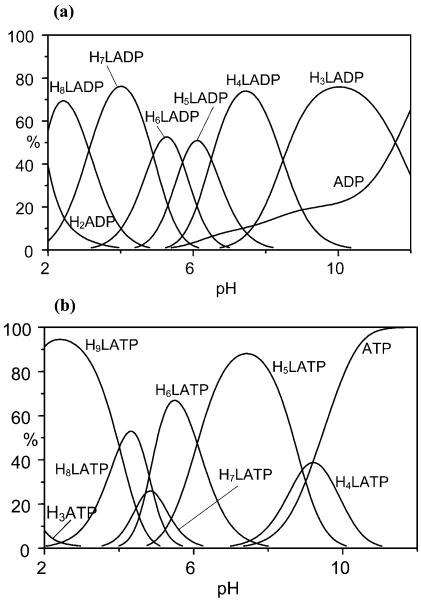


Figure 3. Distribution diagrams for the systems H_2L^{2+}/ADP (a) (0.1 mol dm^{-3} NMe_4Cl, 298.1 K, $[H_2L^{2+}]$ = [ADP] = 1.10^{-3} mol dm^{-3}) and H_2L^{2+}/ATP (b) (0.1 mol dm^{-3} NMe_4Cl, 298.1 K, $[H_2\mathbf{L}^{2+}]$ = [ATP] = 1×10^{-3} mol dm^{-3}).

Plots of the pH dependence of the logarithms of the conditional constants (Figure 4) for the different systems show that all five substrates form remarkably stable complexes with the receptor in a wide pH range. In all cases, the stability slightly increases from alkaline to slightly acidic pH values, with a maximum between pH 3.5 and 4.5, and then decreases markedly at higher pH values. In the alkaline and slightly acidic pH region, the substrates are in their less protonated and highly charged forms, and therefore an increasing number of protonated ammonium functions may enhance the receptor ability to give electrostatic and hydrogen bonding interactions with the anionic substrates, thus lead-

ing to the observed increasing stability from pH 10 to about 4. The marked decrease of the K_{eff} values at more acidic pH values can be reasonably attributed to the formation in solution of highly or fully protonated forms of the substrates, featured by a low or null negative charge.

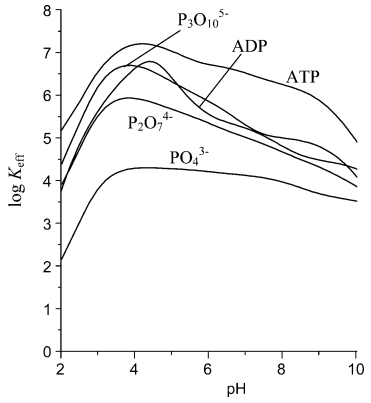


Figure 4. Logarithms of the effective stability constants for the systems $PO_4^{3-}/P_2O_7^{4-}/P_3O_{10}^{5-}/ADP^{3-}/ATP^{4-}$: $H_2\mathbf{L}^{2+}$ ($[H_2\mathbf{L}^{2+}]$ = [substrates] = 1×10^{-3} mol dm^{-3}).

Comparing the binding ability of L towards the different substrates, the stability of the adducts increases in the order phosphate < diphosphate < triphosphate ≈ ADP < ATP. This sequence can be only partially explained in terms of electrostatic and hydrogen bonding interactions between the receptor and the different phosphate anions. Actually, the higher K_{eff} values observed for triphosphate with respect to di- and monophosphate can be reasonably attributed to the large negative charge gathered on triphosphate at a given pH (for instance at pH 4 the three inorganic anions are mainly present in solution as $H_2PO_4^-$, $H_2P_2O_7^{2-}$ and $H_2P_3O_5^{3-}$, respectively) as well as to the larger number of phosphate groups potentially available to interact via hydrogen bonding with the receptor.

The adducts with ADP and ATP display a somewhat higher stability than those with tri- and diphosphate which cannot be interpreted considering only electrostatic and hydrogen bonding interactions involving the phosphate chain of the anions. In fact, the nucleotides are present in solution at pH 4 as H_2ATP^{2-} and $HADP^{2-}$, i.e., with negative charge lower (H_2ATP^{2-}) or equal ($HADP^{2-}$) to that of tri- and diphosphate, respectively.

This could indicate that, in addition to the electrostatic interactions between the phosphate chain and the polyammonium receptor, other effects (hydrogen bond interactions between adenine N atoms and/or hydroxy groups of nucleotides and ammonium functions, hydrophobic and/or π-stacking interactions between the heteroaromatic moieties and cation-π system interactions between the charged ammonium group and adenine) may contribute to the stability of the adducts.

To elucidate the structural features of adducts with ATP and ADP, anion coordination has also been followed by recording ^{31}P NMR spectra on solutions containing receptors and substrates in 1:1 molar ratio at different pH values. Figures 5 and 6 show the pH dependence of the ^{31}P chemical shifts of the phosphate groups of ATP and ADP in the presence of 1 equiv. of the receptor at different pH values, together with those of free ATP and ADP. A list of the complexation-induced ^{31}P chemical shifts (CIS) for the ATP-$H_2\mathbf{L}^{2+}$ and ADP-$H_2\mathbf{L}^{2+}$ systems at pH 6 and 6.5, respectively, is reported within the Supporting Information (Table S5).

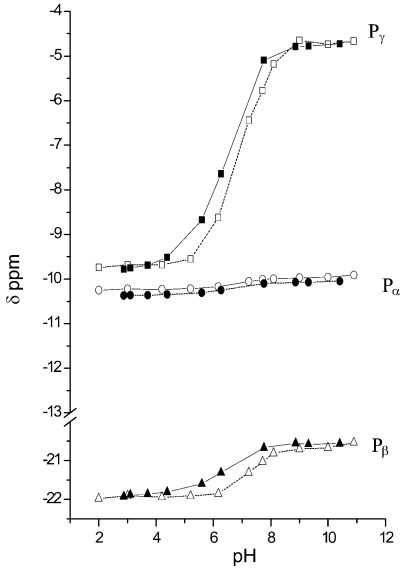


Figure 5. Experimental ^{31}P chemical shifts of free ATP (·····, empty symbols) and of ATP in the presence of $H_2\mathbf{L}^{2+}$ (——, filled symbols) as a function of pH ([ATP] = $[H_2\mathbf{L}^{2+}] = 1 \times 10^{-3}$ mol dm^{-3}, 298.1 K).

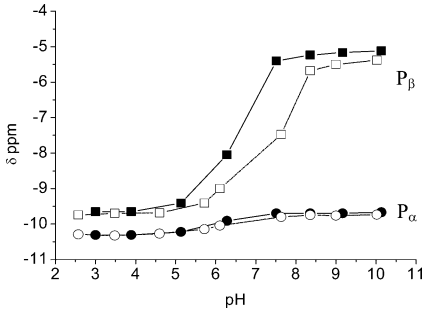


Figure 6. Experimental ^{31}P chemical shifts of free ADP (·····, empty symbols) and of ADP in the presence of $H_2\mathbf{L}^{2+}$ (——, filled symbols) as a function of pH ([ADP] = $[H_2\mathbf{L}^{2+}] = 1 \times 10^{-3}$ mol dm^{-3}, 298.1 K).

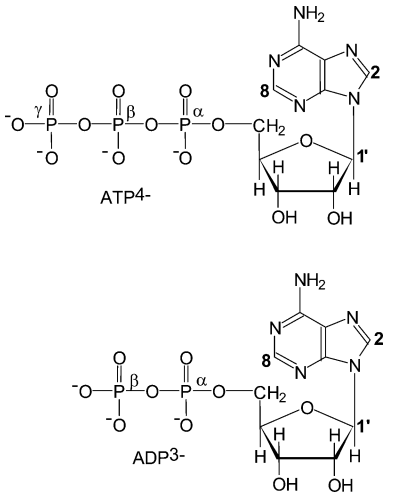
The plots in Figures 5 and 6 indicate that coordination of substrates produces significant variations in the ^{31}P chemical shifts, as already observed for analogous associated species with other polyammonium macrocyclic receptors.[4,5]

In the case of the adducts with ATP, Figure 5 shows a downfield shift upon complexation of the signals of the phosphate groups P_γ and, at a less extent, P_β, while the chemical shift of P_α is almost not influenced by the interaction with the receptors, suggesting that in ATP binding the ammonium functions of the receptor mainly interact with

the two contiguous phosphate groups of nucleotides P_β and P_γ. In the case of ADP, the resonance of the terminal phosphate group P_β shifts downfield in the presence of the receptor, while the chemical shift of the P_α is almost not influenced by the interaction, indicating that in the adducts with ADP the polyammonium receptor interacts mainly with the terminal phosphate group P_β of the nucleotide. In both ATP and ADP complexation, however, the variations of chemical shifts are pH dependent, being greater in the pH range 9–4, in keeping with the potentiometric study of these systems, which has shown that large amounts of the 1:1 receptor/substrate adducts are formed from slight alkaline to acidic pH values (Figure 3), i.e., in the pH region where highly protonated species of the receptor and anionic species of ADP or ATP are simultaneously present in solution.

The ^{31}P NMR spectroscopic data confirm the important role played in the formation of the adducts by the phosphate chains, which can interact via electrostatic forces and hydrogen bonding with the ammonium groups of the receptor.

^1H NMR spectra carried out on solutions containing the receptor and ADP or ATP can provide evidences for the participation of π-stacking interactions in the stabilization of the adducts with receptor (for labelling, see ligand drawing in Figure 1 and Scheme 1). The pH dependence of the signals of the adenine protons H2 and H8 and of anomeric proton H1′ the for both nucleotides in absence and in the presence of receptor is displayed in Figure 7, while the complexation-induced ^1H chemical shifts (CIS) for the nucleotides-$H_2\mathbf{L}^{2+}$ systems at a fixed pH value are reported within the Supporting Information (Table S6). Throughout the pH range in which interaction occurs, upfield displacements are observed for these signals of both nucleotides in the presence of the receptor. However, the variation of chemical shifts observed upon ATP and ADP complexation by the receptor are generally rather low (for instance CIS value of –0.07, –0.12, and –0.12 ppm are measured for the signals of the H2, H8 and H1′ protons of ATP at pH 6) than those observed for ATP or ADP coordination by polyammonium macrocycles containing phenylene spacers.[5] Remarkably higher displacements of the resonances (more than 1 ppm) for the H atoms of the adenine moiety are usually found when π-stacking interactions give an important contribution to complex formation, as in the case of polyammonium macrocycles containing large heteroaromatic systems, such as phenanthroline.[17b] Conversely, the present



Scheme 1.

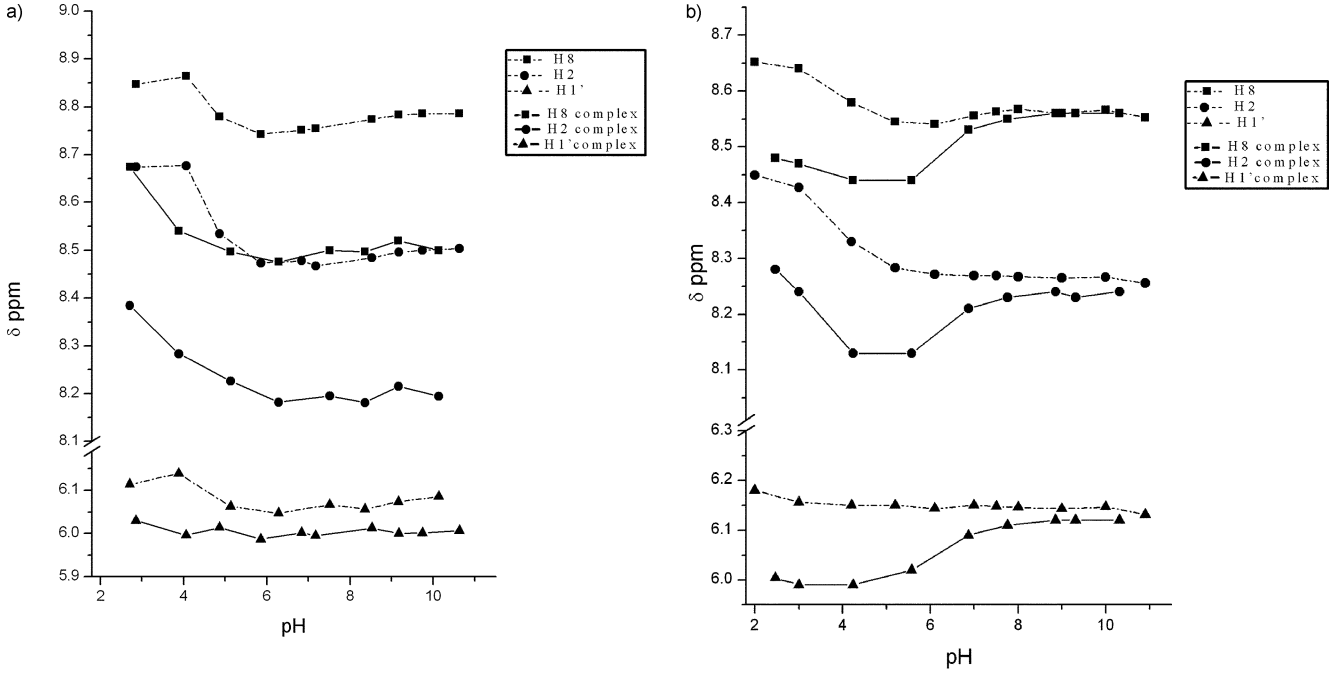


Figure 7. Experimental ^1H chemical shifts for the aromatic protons of free ADP (----) and of ADP in the presence of $H_2\mathbf{L}^{2+}$ (——) (a) and for the aromatic protons of free ATP (----) and of ATP in the presence of $H_2\mathbf{L}^{2+}$ (——) (b).

data account for a rather weak π-stacking interaction occurring between the adenine unit of nucleosides and pyridine moiety of the ligand.

Molecular Modelling

MD simulations were performed on the protonated receptors H_hL^{h+} (h = 3, 4 and 6) in vacuo and by using an implicit water model. The analysis of each MD trajectory reveals no significant changes in the overall shape of the cation within every simulation. However, a comparison of the average conformations resulting from the six MD protocols highlights the different shapes adopted by the receptor depending on its charge and on the nature of the surrounding medium. In particular, depending on the molecular charge and the solvent polarity, the two lateral reinforced macrocyclic systems arrange in a different way with respect to each other and to the central macrocycle, thus giving different shapes to the receptor. The relative distances between the geometric centroids of the three [12]aneN_4 rings and the centroid of the overall molecule (C_{MT}) were computed in order to quantify the differences in the overall molecular shape. For example in vacuo, by increasing the molecular charge, the side arms spread out progressively: 6.4, 7.6 and 12.0 Å are the mean distances from C_{MT} and the centroids of the terminal reinforced rings in H_3L^{3+}, H_4L^{4+} and H_6L^{6+}, respectively. The H_3L^{3+} receptor has on average a sort of flat calyx-like shape, with the central macrobicyclic ring at the base and the terminal reinforced [12]aneN_4 rings in a head-to-tail relative arrangement (Figure 8, a). By adding positive charges the side arms unfold progressively: the H_6L^{6+} species is completely open (a kind of flat cylinder, Figure 8, b) and, at variance with the previous two species (H_3L^{3+} and H_4L^{4+}) shows three distinct, equally charged and accessible binding sites (i.e., the tetraaza rings) for the guest. As expected, the increase of the solvent polarity

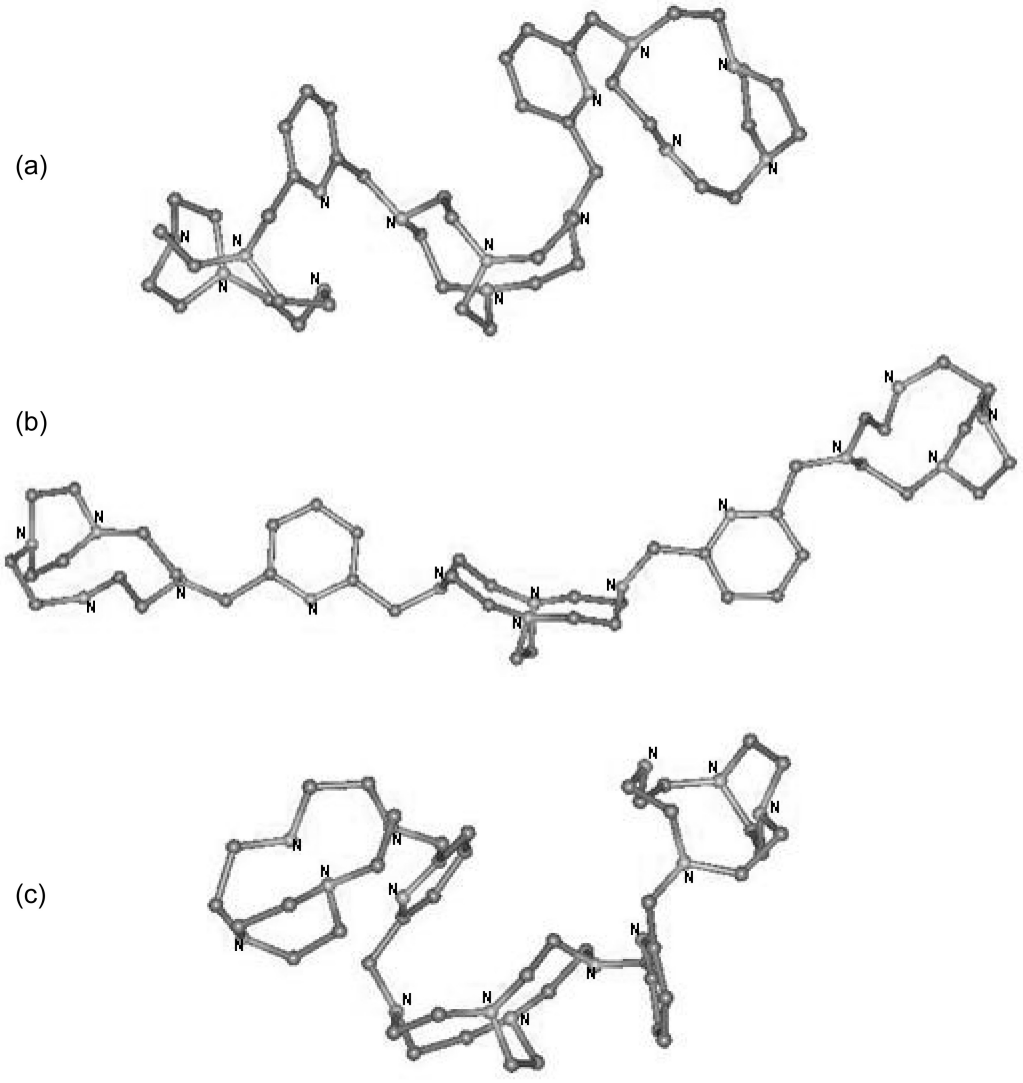


Figure 8. a) View of the minimum energy conformation of the H_3L^{3+} receptor in vacuo; b) view of the minimum energy conformation of the H_6L^{6+} receptor in vacuo; c) view of the minimum energy conformation of the H_6L^{6+} receptor in the implicit water model environment.

makes the positively charged side arms close again about the central reinforced macrocyclic ring almost facing each other and, as a consequence, all the cations shape a sort of calyx (see for example $H_6\mathbf{L}^{6+}$ in Figure 8, c). Now the computed inter-centroid distances are: 4.2, 5.2 and 5.2 Å in $H_3\mathbf{L}^{3+}$, $H_4\mathbf{L}^{4+}$ and $H_6\mathbf{L}^{6+}$, respectively.

In summary the $H_3\mathbf{L}^{3+}$ cation appears the most crowded in both the environments: its potential binding sites are well inside the molecular framework.

The interaction of the minimised protonated forms of the receptor with ATP and ADP was then analysed. In the minimisation procedure, we chose the protonated forms of ATP and ADP present in aqueous solutions in the pH range where the $H_3\mathbf{L}^{3+}$, $H_4\mathbf{L}^{4+}$ and $H_6\mathbf{L}^{6+}$ are formed and therefore we analysed the adducts $[(H_3\mathbf{L})(ADP)]$, $[(H_3\mathbf{L})(HATP)]$, $[(H_4\mathbf{L})(ADP)]^+$, $[(H_4\mathbf{L})(HATP)]^+$, $[(H_6\mathbf{L})(HADP)]^{4+}$ and $[(H_6\mathbf{L})(HATP)]^{3+}$. The interaction of $H_3\mathbf{L}^{3+}$ and $H_4\mathbf{L}^{4+}$ with the two nucleotides was first analysed, docking the nucleotide anionic species ADP^{3-} and $HATP^{3-}$ into the $H_3\mathbf{L}^{3+}$ and $H_4\mathbf{L}^{4+}$ receptors. As already mentioned, in the lowest energy conformation of the $H_3\mathbf{L}^{3+}$ cation, both in vacuo and in the polar medium, the acidic protons point inside the receptor framework and are involved in some intramolecular H-bonds, preventing any hydrogen bond interactions between the nucleotides and the receptor (the sum of the hydrogen and oxygen Van der Waals radii, that is 2.7 Å,[31] was considered as threshold to spot H-bond interactions). The inspection of the retained poses, with the term poses we refer to different positioning and orientation of the anions with respect to the binding site of the ligand,[32] shows that in vacuo both the anions wrap around the terminal reinforced macrocycle bearing the bipositive charge, while in the polar medium the nucleo-

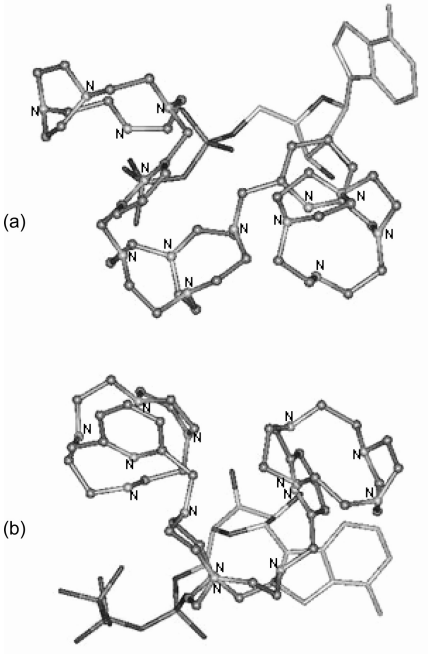


Figure 9. a) View of the best pose of the $H_4\mathbf{L}^{4+}/ADP^{3-}$ adduct in the implicit water model environment; b) view of the best pose of the $H_4\mathbf{L}^{4+}/HATP^{3-}$ adduct in the implicit water model environment.

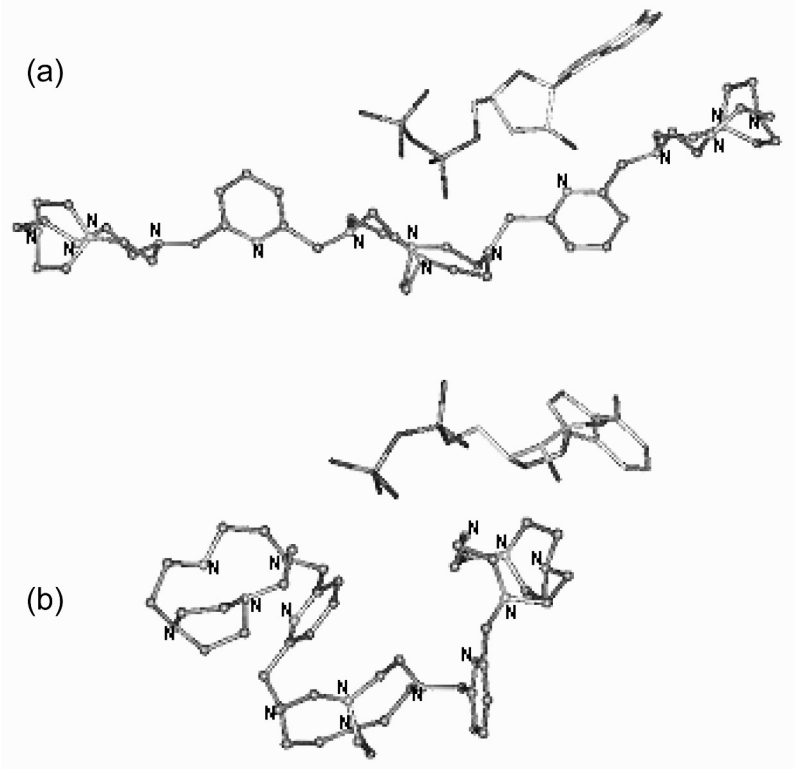


Figure 10. a) View of the best pose of the $H_6\mathbf{L}^{6+}/HADP^{2-}$ adduct in vacuo; b) view of the best pose of the $H_6\mathbf{L}^{6+}/HADP^{2-}$ adduct in the implicit water model environment.

tides stand on the top of the receptor cavity (Figure S3). In the case of the H_4L^{4+}, the saved poses, both in vacuo and in the polar medium, show the smaller ADP anion anchored (only intermolecular NH···O distances less than the sum of the oxygen and hydrogen Van der Waals radii were considered) to a NH_2^+ group invariably thanks to hydrogen bond interactions involving the oxygen atoms of the P_β phosphate group (Figure 9, a). In the case of ATP, the two contiguous P_β and P_γ phosphate groups of the $HATP^{3-}$ species are involved in the interaction with the protonated secondary nitrogens of H_4L^{4+} (Figure 9, b). Finally, in vacuo poses reveal that both the nucleotides stand on the terminal reinforced tetraaza ring with the aromatic moiety on the top of the central macrobicycle, while in the simulated solvent both of them wrap about the terminal reinforced cyclen (Figure S4).

Results from the docking of the $HADP^{2-}$ and $HATP^{3-}$ species to the H_6L^{6+} cation evidence the leading role of the acidic H atoms of the central macrobicycle in the interaction with the tested guests in vacuo. In fact in the open and outstretched conformation of the H_6L^{6+} cation, they are facing each others and point towards the upper surface of the tetraaza ring allowing the formation of strong hydrogen bond interactions (NH···O distances less than 2.0 Å) with the oxygen atoms provided by the phosphate groups of both the nucleotides. The latter are on the top of the central tetraaza ring and in most of the saved poses the oxygen atoms bound to P_α of $HADP^{2-}$ and P_β of $HATP^{3-}$ act as bifurcated acceptors towards the acidic hydrogens of the central macrobicycle (part a of Figure 10 and Figure S5). In the polar medium, instead, both the anions interact via H-bonds with the NH_2^+ group of a terminal tetraaza ring and stand on the top of the calyx (Figure 10, b). Analyses of poses (Figure S6) reveal that in both the nucleotides the H-bond acceptors are provided by the terminal phosphate group P_β for $HADP^{2-}$ and by the two phosphate moieties P_β and $P\gamma$ for $HATP^{3-}$ and that in some cases the acidic proton acts as bifurcated donor.

Conclusions

The present tris-macrocycle **L** behaves as "double" proton sponge, giving rise in aqueous solution to a H_2L^{2+} species which cannot be deprotonated even at strongly alkaline pH values. The two acidic protons are localized in two lateral reinforced macrocyclic units. Further protonation of H_2L^{2+} affords a H_4L^{4+} species, where each lateral tetraamine macrocycle is diprotonated. The central macrocyclic units is involved in proton binding only in the species with higher protonation degree. These positively charged forms of the receptor give stable 1:1 adducts with inorganic phosphate anions, ADP and ATP. The stability of the adducts is mainly determined by electrostatic and hydrogen bonding interactions. Due to the particular calyx-like shape assumed by the receptor in aqueous solutions, only the lateral macrocyclic moieties seem to be involved in anion binding, while the central tetramine unit is shielded from the interaction with the anionic substrates. In nucleotide binding, both ^{31}P NMR experiments and MD calculations, carried out by using an implicit water model, show that the lateral macrocyclic units interact mainly with the terminal and central phosphate groups of ATP and with the terminal phosphate group of ADP. Conversely, the adenine moiety of nucleotides seems to play a minor role in the stabilization of the adducts.

Experimental Section

Materials: The sodium salts of ATP and ADP and $Na_2HPO_4{\cdot}2H_2O$, $Na_4P_2O_7{\cdot}10H_2O$, $Na_5P_3O_{10}{\cdot}6H_2O$, employed in the potentiometric measurements, were purchased from Merck.

Synthesis of L

Cyclen-glyoxal **2** was synthesised as described previously.[25]

Compound 3: To a stirred solution of 2,6-bis(bromomethyl)pyridine (4 g; 15.1 mmol) dissolved in 15 mL of dry acetonitrile, a solution of cyclen-glyoxal **2** (0.75 g; 3.8 mmol) in 10 mL of dry acetonitrile was added dropwise. The mixture was stirred at room temperature for a week. The precipitate was collected by filtration, washed with diethyl ether and dried in vacuo, giving **3** as a white powder with 90% of yield. ^{13}C NMR (100.62 MHz; [D_6]DMSO, 298 K): δ = 33.6, 42.4, 46.4, 55.5, 59.6 (αCH_2), 60.4 (αCH2-Py), 76.4 (CH-aminal), 122.2, 123.4, 136.5, 148.4, 159.8 (CH-Ar) ppm. MALDI-TOF (H_2O): *m/z* 720.94 $[M + H]^+$. $C_{24}H_{32}Br_4N_6{\cdot}H_2O$ (742.19): calcd. C 38.73, H 7.04, N 11.29; found C 39.01, H 7.12, N 11.61.

Compound 4: Compound **3** (1.5 g; 2 mmol) was added to a solution of bis-aminal **2** (1.2 g; 6.2 mmol) in 25 mL of dry DMF. The mixture was stirred at room temperature for 10 d. The precipitate was collected by filtration, washed with diethyl ether. The solid was dissolved in water; a subsequent addition of ethanol leads to a precipitate which is filtered and dried in vacuo, at 60 °C, giving **4** as a white powder with 70% of yield. ^{13}C NMR (100.62 MHz; D_2O; 298 K): δ = 45.5, 46.6, 49.4, 50.5, 50.8, 51.1, 51.2, 54.2, 58.6, 63.6, 64.1, 65.3, 65.3, 65.6 (αCH_2), 74.5, 81.3, 86.3, 86.5 (Ca), 131.6, 131.8, 143.4, 143.5, 151.4, 151.6, 151.6, 152.2 (Car) ppm. MALDI-TOF (H_2O): *m/z* 1082.2 $[M + H]^+$. $C_{42}H_{64}Br_4N_{14}{\cdot}2H_2O$ (1120.71): calcd. C 45.83, H 9.44, N 17.01; found C 45.51, H 9.22, N 17.31.

Compound L: A large excess of $NaBH_4$ (24 equiv.) was added in small portions over 1 h to a stirred solution of polyammonium salts **4** in absolute ethanol (1 g in 60 mL). The mixture was allowed to stir at room temperature for one week. After cooling at 0 °C and aqueous HCl (2 M) addition until pH ≈ 3–4, the resulting mixture was evaporated to dryness. The resulting white solid was then dissolved in a small quantity of water and potassium hydroxide pellets were added until basic medium. The aqueous phase was then evaporated to dryness and the residue was extracted with chloroform (3 × 50 mL). The combined organic phases were finally evaporated to give L in 78% of yield (0.590 g). ^{13}C NMR (75.47 MHz. $CDCl_3$; 298 K): δ = 46.7, 48.2, 48.4, 50.0, 51.8, 52.0, 55.8, 56.0, 56.7, 56.9, 57.2 (αCH_2); 61.0, 61.2 (αCH_2Py), 119.9, 136.2, 136.6, 159.0, 159.2 (CAr) ppm. MALDI-TOF ($CHCl_3$, Dithranol): *m/z* 801.6 $[M + H]^+$.

Hydrochloride derivatives were obtained by addition of concentrated aqueous HCl to an ethanolic solution of ligand at 0 °C. The resulting white precipitate was filtered, washed with absolute ethanol and dissolved in a solution of 6 M HCl. After 2 h at 80 °C, solvent was evaporated to give a white powder which was dried under vacuum at 80 °C for 24 h. $C_{44}H_{76}N_{14}{\cdot}8HCl{\cdot}H_2O$ (1106.46):

calcd. C 43.87, H 8.04, N 16.28, Cl 23.84; found C 43.81, H 7.72, N 16.02, Cl 24.12.

Potentiometric Measurements: All pH metric measurements (pH = –log[H^+]) were carried out in degassed 0.1 mol dm^{-3} NMe_4Cl solutions, at 298.1 ± 0.1 and 313.1 ± 0.1 K, by using equipment and procedure which have been already described.[33] The combined Ingold 405 S7/120 electrode was calibrated as a hydrogen concentration probe by titrating known amounts of HCl with CO_2-free NMe_4OH solutions and determining the equivalent point by the Gran's method[34] which allows to determine the standard potential $E°$, and the ionic product of water [pK_w = 13.83(1) and pK_w = 13.40(1) at 298.1 K and 313.1 K, respectively, in 0.1 mol dm^{-3} NMe_4Cl]. In all the experiments the ligand concentration [**L**] was about 1×10^{-3} mol dm^{-3}. In the anionic coordination experiments the anion concentration was varied over the range [**L**] ≤ [anion] ≤ 2[**L**]. At least four measurements (about 150 data points each one) were performed in the pH range 2.3–12.0 for all systems.

The e.m.f. data were treated by means of the computer program HYPERQUAD[28] which furnished the equilibrium constants reported in Tables 1, 2, and 3.

NMR Spectroscopy: ^{1}H spectra in D_2O solution at different pH values were recorded at 298.1 K and 313.1 K in a Varian 300 MHz spectrometer. In ^{1}H NMR spectra peak positions are reported relative to DSS. ^{1}H-^{1}H 2D correlation experiments were performed to assign the signals. Small amounts of 0.01 mol dm^{-3} NaOD or DCl solutions were added to a solution of **L** to adjust the pD. The pH was calculated from the measured pD values using the relationship pH = pD – 0.40.[35]

Molecular Modelling Procedures: The three $H_3\mathbf{L}^{3+}$, $H_4\mathbf{L}^{4+}$ and $H_6\mathbf{L}^{6+}$ differently protonated species of the receptor and their adducts with different anionic species of the nucleotides (ADP^{3-}, $HATP^{3-}$ and $HADP^{2-}$) were taken into account in the modelling studies. In all cases the speciation of the modelled adducts was retrieved from the potentiometric data. Three were the objectives of the modelling study: i) to assess the role of the degree of protonation in determining the conformational behaviour of **L**; ii) to compare the complexation ability of the same protonated form of the receptor $H_h\mathbf{L}^{h+}$ (h = 3, 4) towards equally charged nucleotides (ADP^{3-}, $HATP^{3-}$); iii) to test how the charge of the substrate ($HADP^{2-}$ vs. $HATP^{3-}$) affects the interaction with the receptor ($H_6\mathbf{L}^{6+}$). Due to the lack of single crystals of **L** or $H_h\mathbf{L}^{h+}$ suitable for X-ray diffraction, the starting 3D framework of the receptor was obtained by assembling appropriate molecular fragments retrieved from the Cambridge Structural Database (CSD; v.5.28).[36] The protonation sites of each $H_h\mathbf{L}^{h+}$ species were identified on the basis of the ^{1}H NMR experiments performed at different pH values: in $H_3\mathbf{L}^{3+}$ the acidic protons are bound to the two reinforced N atoms N1 and N1′and to the secondary N3; $H_4\mathbf{L}^{4+}$ features the additional proton on N3′; in $H_6\mathbf{L}^{6+}$ the nitrogen atoms N6 and N6′of the central ring bear the further hydrogen ions.

Each starting model $H_h\mathbf{L}^{h+}$, roughly improved by an energy minimization procedure, underwent molecular dynamics (MD) simulations (time step: 1fs, equilibration time: 100 ps, production time: 1000 ps, T = 300 K) in order to explore its potential energy surface both in vacuo and in water, the latter simulated by using a distance dependent dielectric constant. Then energy minimization procedures (by using the steepest descent and conjugate gradient algorithms) were applied in order to obtain the starting geometries for the subsequent docking protocols. The programs used for the MD and the energy minimization were the simulation protocols Standard Dynamics Cascade and Minimization implemented in Accelrys Discovery Studio 1.7.[37] The starting geometries for the nucleotides$^-$ were retrieved from the CSD. Atomic charges were calculated by the Gasteiger method. Docking simulations were performed at 300 K both in vacuo and in the simulated water solvent by using the protocol Ligand Fit implemented in Discovery Studio. In all adducts the doubly charged reinforced tetraaza ring of the $H_h\mathbf{L}^{h+}$ species was taken into account for the receptor-substrate interaction. In addition, also the central charged macrobicycle of $H_6\mathbf{L}^{6+}$ was considered for the interaction with the guests in the docking protocols performed in vacuo.

Different substrate conformations were generated during the docking procedure by means of the Monte-Carlo method. In-situ ligand minimization was performed by means of the Smart Minimizer algorithm, the number of saved poses was 40. The Force Field used for all the simulations was CHARMm.[38]

Supporting Information (see also the footnote on the first page of this article): Protonation constants of substrates, stepwise stability constants of the adducts, CIS values of the ^{31}P and ^{1}H NMR signals of ADP and ATP in the presence of the receptor, distribution diagram of protonated species of L at 298.1 K, distribution diagrams of **L** with inorganic phosphate, best poses of the $H_3\mathbf{L}^{3+}$/ADP^{3-}, $H_3\mathbf{L}^{3+}$/$HATP^{3-}$ and $H_6\mathbf{L}^{6+}$/$HATP^{3-}$ adducts in vacuo and in the implicit water model environment; best poses of the $H_4\mathbf{L}^{4+}$/ADP^{3-} and $H_4\mathbf{L}^{4+}$/$HATP^{3-}$ adduct in vacuo.

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