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Studies on the interaction of phosphate anions with *N*-functionalised polyaza[*n*]paracyclophanes: the role of *N*-methylation

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The synthesis and interaction of *N*,*N*′,*N*″,*N*‴-tetramethyl-2,6,9,13-tetraaza[14]paracyclophanes (**B323Me$_4$**) with nucleotides and inorganic phosphates is described. An easy methodology is used to define thermodynamic selectivity. The interaction of B323Me4 and ATP has been monitored by ^{1}H, ^{31}P NMR and by molecular mechanics, ruling out the possibility of the participation of π-stacking interactions. Results show that *N*-methylation is accompanied by a strong increase in the interaction of the resulting macrocycle with ATP.

Polyazacyclophanes are versatile molecules able to complex either organic or inorganic substrates.[1] The presence of nitrogen donor atoms provides a way for the interaction with transition metal cations, but, at the same time, those receptors are able to strongly interact with anions in their protonated forms. On the other hand, the aromatic spacer plays an essential role increasing the hydrophobicity of the site, incorporating potential π interactions and precluding the involvement of all the nitrogen donors in the coordination to a single metal center. In polyazamacrocycles, the presence of nitrogen atoms facilitates the introduction of a variety of different arms through their *N*-functionalisation. Tuning the nature of the *N*-attached arms, for example introducing carboxylic arms or hydrophobic substituents, represents a simple way to improve selectivity towards some given guests and to incorporate specific activities, so we can consider those macrocycles as molecules with smart functions.[2] During the last years, there has been an increasing interest in the study and complexation of anionic species, according to the fact that a large number of the substances and cofactors engaged in biological processes are anions.[3] In this context, polyaza[*n*]paracyclophanes have shown interesting properties in their interaction with different anions.[4]

Here we report on the synthesis and on the interaction studies of *N*,*N*′,*N*″,*N*‴-tetramethyl-2,6,9,13[14]paracyclophane (**B323Me$_4$**) with different organic and inorganic phosphate anions. The interaction of polyazamacrocycles with phosphate anions is of great interest due to the important role that phosphates play in biological processes.[5] *N*-Methylation of B323 can provide important differences in the properties of **B323Me$_4$** with regards to the parent macrocycle. The increase in hydrophobicity, the changes in acid–base properties and the modification of conformational equilibria can strongly affect the expected interaction of this receptor with different guests. In this work, we have focused our attention on the interaction of **B323Me$_4$** (**2**) with the nucleotides ATP, ADP and AMP and the inorganic anions pyrophosphate and tripolyphosphate.

Results and discussion

Synthesis of receptors

The synthesis of the precursor polyaza[*n*]paracyclophane (**B323**, **1**) was carried out according to literature procedures.[1a] Permethylation of this polyazamacrocycle was obtained using an Eschweiler–Clarke procedure,[6] affording quantitative yields of **2** (see Scheme 1).

The same synthetic procedure was used to prepare, also in quantitative yields, other *N*-methylated macrocycles such as **B222Me$_4$** (**4**) and **D222Me$_4$** (**6**) from B222 (**3**) and **D222** (**5**) (see Chart 1).

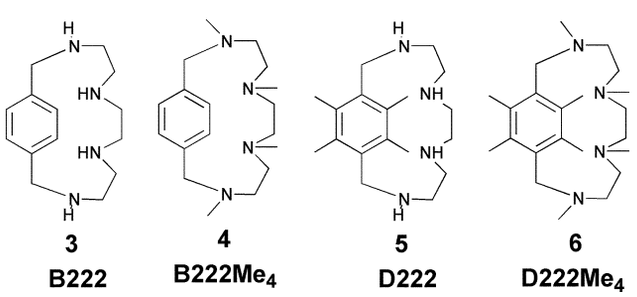
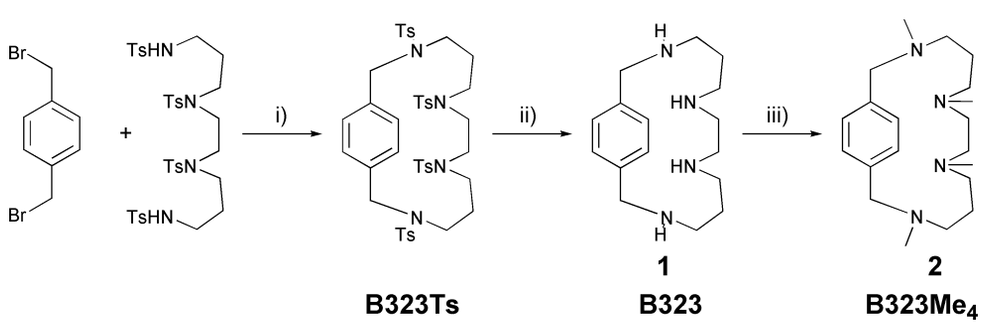


Chart 1



Scheme 1 Synthesis of **B323Me$_4$**: i) K_2CO_3, CH_3CN; ii) Na/Hg, Na_2HPO_4; iii) HCOOH, H_2CO.

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Table 1 Logarithms of the stepwise protonation constants for the protonation of different polyazacyclophanes[a]

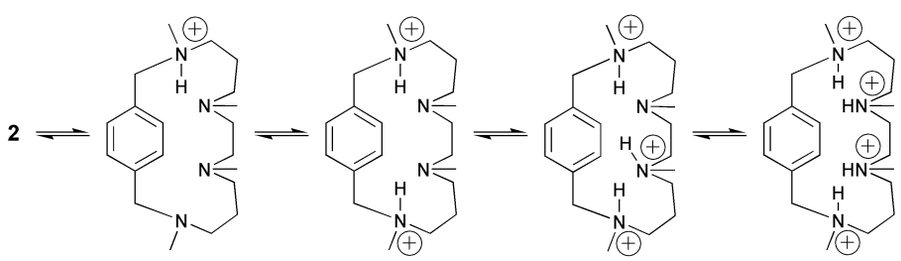
Reaction[b]	B323Me$_4$	B323	D323	D323Me$_4$	B222	B222Me$_4$
H + L = HL	8.99 (3)[c]	9.93[d]	10.54[e]	11.15 (4)	9.39 (2)	9.07 (4)
H + HL = H_2L	8.22 (3)	9.09	9.32	9.12 (4)	8.45 (2)	7.96 (4)
H + H_2L = H_3L	6.59 (3)	7.44	7.44	7.34 (4)	5.38 (2)	3.88 (4)
H + H_3L = H_4L	3.69 (4)	3.61	3.59	3.84 (5)	2.51 (1)	<2.0
Log β	27.49	30.07	30.89	31.45	25.73	<22.91

[a] Determined in 0.15 mol dm^{-3} NaCl at 298.1 K. [b] Charges omitted for clarity. [c] Values in parentheses are standard deviations in the last significant figure. [d] Taken from reference 1b. [e] Taken from reference 10.

Table 2 Logarithms of the stepwise stability constants for the interaction of **B323Me$_4$ (2)** with different nucleotides and phosphate anions[a]

Reaction[b]	ATP	ADP	AMP	PYR	TPP
H_4L + H_2A = H_6LA	5.40 (4)[c]	3.94 (3)	—	3.83 (5)	4.10 (4)
H_3L + H_2A = H_5LA	6.10 (3)	4.69 (3)	3.71 (5)	3.98 (5)	4.35 (4)
H_3L + HA = H_4LA	5.59 (3)	4.53 (2)	4.32 (2)	4.15 (3)	4.40 (4)
H_2L + HA = H_3LA	—	—	—	4.04 (3)	4.19 (4)
H_3L + A = H_3LA	5.39 (3)	4.42 (3)	4.44 (3)	—	—
HL + HA = H_2LA	—	—	—	—	4.22 (4)
H_2L + A = H_2LA	4.73 (2)	4.01 (1)	4.08 (3)	3.97 (3)	4.18 (4)
HL + A = HLA	4.24 (4)	3.67 (3)	3.82 (4)	3.70 (4)	3.76 (6)

[a] Determined in 0.15 mol dm^{-3} NaCl at 298.1 K. [b] Charges omitted for clarity. [c] Values in parentheses are standard deviations in the last significant figure.



Scheme 2 Preferential protonation sites for the successive protonation steps.

Protonation studies

Acid–base properties of compound **2** were studied by the use of pH-metric titrations, as the first step for the analysis of its properties as a receptor. All the titrations were carried out as has been fully described,[7] at 298.1 K using NaCl 0.15 M to maintain a constant ionic strength. The program PASAT was used for data acquisition.[8] The program HYPERQUAD was employed for the analysis of those data and stability constant calculations, and the program HYSS was used to obtain the distribution diagrams.[9] The stability constants for the protonation of **B323Me$_4$** obtained in this way are presented in Table 1. For comparison, the stability constants for the protonation of related polyazacyclophanes **1** and **3**–**6** have been also included.[10]

As can be seen in the table, stepwise protonation constants for **B323Me$_4$** follow similar trends to those found for related receptors such as **B323**,[7,10] with the presence of two large constants for the two first protonation steps, an intermediate one and a smaller one corresponding to the last protonation step. The *N*-methylation is accompanied by a slight decrease in the basicity of **B323Me$_4$** as compared with that of **B323**. This decrease in basicity affects the first three protonation steps and is not very important for the last one. The same is true for the receptor **B222Me$_4$**, for which the value of the global basicity constant is about 2.5 logarithmic units smaller than that for **B222**. Only in the case of **D323Me$_4$** is the basicity comparable with that of the parent compound **D323**. In this case, the first protonation constant is larger for **D323Me$_4$** than for any other of the macrocycles in Table 1. It has to be taken into account that **D323** itself contains four methyl groups on the aromatic subunit. According to the former data, the increase in hydrophobicity that accompanies *N*-methylation, and conformational factors that could make minimisation of electrostatic repulsion difficult, clearly compensate for the favourable electronic effects provided by the introduction of methyl groups on the nitrogen atoms

In the case of **B323Me$_4$**, ^{1}H and ^{13}C NMR spectra at variable pH give information about its average protonation (see Scheme 2).[11] For instance, the benzylic protons experience a significant downfield shift from pH 12 to 4, the region corresponding to the uptake of the first two protons, remaining essentially unchanged at lower pH values. On the contrary, the downfield shift for the protons of the central ethylene bridge is more pronounced between pH 4 and 2, the region involving the third and fourth protonation steps. These NMR data suggest that the first two protonation steps mainly affect the benzylic nitrogen atoms. The same results can be obtained by the use of ^{15}N NMR spectroscopy, the signal of the benzylic nitrogens being essentially shifted downfield (from −340 to −335 ppm) between pH 12 and 4.[12]

According to those data, *N*-methylation of **B323** does not produce significant changes on the protonation scheme of the resulting macrocycle.[10] The same seems to apply to the other *N*-methylated macrocycles prepared (**4** and **6**).

Anion binding studies

The interaction of **B323Me$_4$** with ATP, ADP, AMP, pyrophosphate and tripolyphosphate was also studied by pH-metric titrations. Table 2 gathers the stability constants, determined at 298.1 K in 0.15 mol dm^{-3} NaCl, for the species formed in those systems.

Several features regarding the speciation and the stability of those interactions deserve discussion. First of all, the stoichiometries found for the adducts formed in all systems are always

1 : 1. The protonation degrees of the adducts vary from 1 to 6 for all the species except in the case of AMP for which only pentaprotonated species are formed. Since the receptor and the anions themselves participate in several protonation equilibria that are often overlapped, care has to be taken in order to decide which are the right stepwise equilibria involved in the formation of a given adduct species. To overcome this problem, first of all, one has to consider which are the relevant basicity equilibria for the host and the guest at the pH value for the formation of a given species.[13] Considering all these factors, the stepwise stability constants shown in Table 2 can be proposed as the most feasible ones for the interaction of **B323Me$_4$** with ATP, ADP, AMP, pyrophosphate (PYR) and tripolyphosphate (TPP). In the case of TPP, for the H_2LA species a clear-cut separation cannot be made and this species would be formed either by the reaction of HL and HA or H_2L and A with percentages of participation that would depend on pH.

However using the conditional stability constants a simpler picture of the situation defined according to the following equation can be attained.[13]

$$\log K_s = \Sigma[H_{i+j}AL]/\Sigma[H_iA]\Sigma[H_jL] \qquad (1)$$

This procedure permits a straightforward comparison of the different systems and obtainment of the appropriate selectivity ratios, at the different pH values, just by dividing the corresponding conditional constants. Fig. 1 shows the plots of the conditional stability constants *vs*. pH for the different phosphate anions. In such a graphic, it can be seen that ATP is the substrate exhibiting the largest conditional constants throughout the entire pH range. At acidic pH values, AMP is the species showing the lowest interaction. At basic pH values, AMP, ADP, PYR and TPP present similar conditional constants. In order to explain this trend, the charge of the anions must be the most relevant factor. From the data in Fig. 1, quantitative selectivity ratios can be obtained. Thus, for instance, at pH 4, ligand **2** is 65, 52 and 19 times more selective for ATP than for TPP, AMP and ADP, respectively.

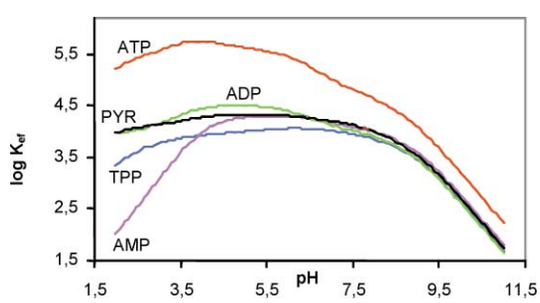


Fig. 1 Representation of the values of the logarithms for the conditional stability constants for the interaction of **B323Me$_4$** with different phosphate anions: ATP-red; ADP-green; PYR-black; TPP-blue; AMP-magenta.

The former data allow an evaluation of the influence of *N*-methylation in the interaction of polyazamacrocycles with anions. Table 3 gathers the stepwise stability constants obtained for the interaction of **B323** and **B323Me$_4$** with ATP.

As it can be seen, the permethylated receptor (**2**) has stability constants that are always two orders of magnitude larger than the corresponding ones for the parent **B323** (**1**). Accordingly, although **B323Me$_4$**, taking into account the data in Table 1, is less basic than **B323**, it interacts much more strongly with ATP species. Fig. 2 shows the plots of the conditional stability constants (calculated using eqn. 1) *vs*. pH for the interaction of **B323** and **B323Me$_4$** with ATP. From those data, it can be inferred that selectivity ratios range from *ca*. 30000 at basic pH values to 50 at acidic pH values, reaching a value of about 100 at the neutral region.

Table 3 Logarithms of the stepwise stability constants for the interaction of ATP with **B323** and **B323Me$_4$**.[a]

Reaction[b]	B323	B323Me$_4$
$H_4L + H_2A = H_6LA$	3.82 (1)[c]	5.50 (4)
$H_3L + H_2A = H_5LA$	4.08 (1)	6.10 (3)
$H_3L + HA = H_4LA$	3.39 (1)	5.59 (3)
$H_3L + A = H_3LA$	2.58 (2)	5.39 (3)
$H_2L + A = H_2LA$	—	4.73 (2)
$HL + A = HLA$	—	4.24 (4)

[a] Determined in 0.15 mol dm^{-3} NaCl at 298.1 K. [b] Charges omitted for clarity. [c] Values in parentheses are standard deviations in the last significant figure.

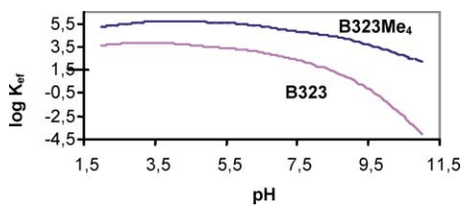


Fig. 2 Representation of the values of the logarithms for the conditional stability constants for the interaction of ATP with **B323Me$_4$** (black) and **B323** (magenta).

We have made use of ^{1}H and ^{31}P nuclear magnetic resonance spectroscopy and molecular mechanics calculations to study the interaction of **B323Me$_4$** with ATP, in order to better understand this behaviour. As has been described before, ^{1}H NMR spectra of the macrocycle displays significant changes in the aliphatic region upon protonation (see Fig. 3). One of the main features for the spectrum of the fully protonated receptor is the presence of a broad band corresponding to the benzylic protons at 4.57 ppm. This can be assigned to a slow rotation of the polynitrogenated chain around the macrocycle.[14] Addition of 1 mol of ATP is accompanied by substantial changes in the signals corresponding to the macrocycle. The benzylic band at 4.57 splits into two broad signals at 4.26 and 4.72 ppm. The same happens for the signal corresponding to the central methylene of the propylenic groups, which is split into two broad signals at 1.99 and 2.25 ppm. This strongly suggests a clear differentiation of the two sides of the macrocyclic plane upon interaction with ATP. A general broadening of the other aliphatic signals is observed and they appear overlapped at 2.9–3.5 ppm. A very significant broadening is also found for the aromatic signal that only experiences a minor change in its chemical shift. If the signals corresponding to ATP are con-

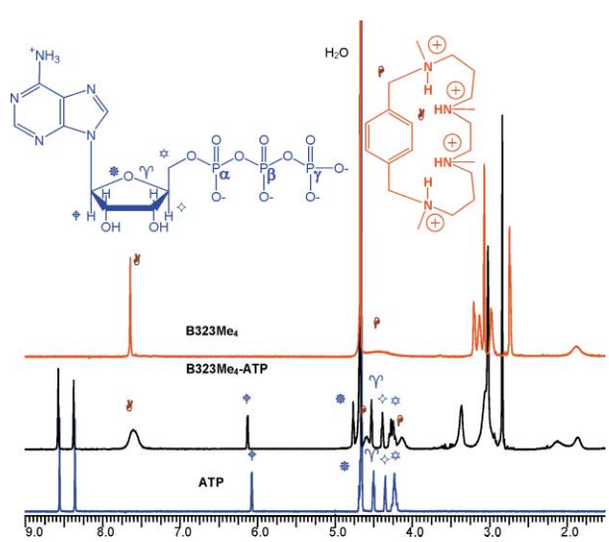


Fig. 3 ^{1}H NMR spectra for ATP, **B323Me$_4$** and the complex **B323Me$_4$**–ATP formed at acidic pH values (pH = 1.6).

sidered, it can be seen that only minor changes occur, with the observation of a slight downfield shift for the protons of the sugar moiety ($\Delta\delta$ < 0.2 ppm). The aromatic signals of ATP remain essentially unchanged. The former observations seem to rule out the existence of π-stacking interactions between the adenine fragment of the ATP and the aromatic subunit of **B323Me$_4$**.

The interaction ATP–**B323Me$_4$** was also followed by ^{31}P NMR. As can be seen in Fig. 4, small changes in the chemical shifts of the three phosphorous atoms are observed along with a significant broadening of the three signals. This suggest that the three phosphate groups would be involved in the binding to the fully protonated **B323Me$_4$**, even if the P_α could be involved at a lower extent. The large broadenings found can be explained through the existence of several complexes in equilibrium, but with the interaction taking place from a single side of the macrocyclic plane.

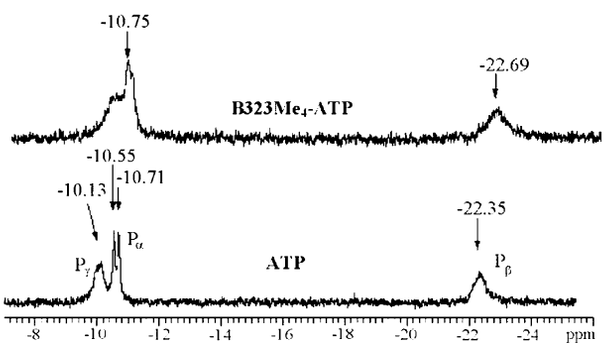


Fig. 4 ^{31}P NMR spectra for ATP and its complex with **B323Me$_4$**.

The use of molecular modelling was very helpful in rationalizing the former observations. Molecular mechanics calculations were carried out using the program MACROMODEL 5.0 with the AMBER force field, as implemented in this package, and with a GB/SA simulation of water as the solvent.[15] The minimum energy conformer found from calculations agrees very well with the experimental data obtained by NMR experiments.

The calculated structure is depicted in Fig. 5 and shows how all the phosphate groups interact with the ammonium groups of the receptor on one side of the plane of the macrocyclic ring. Nevertheless, according to its location and to the calculated distances to the nearest ammonium groups, the P_α phosphate group should interact less strongly than the other two. At the same time, the aromatic adenine subunit is located far away from the aromatic spacer of the polyazacyclophane. Calculations also show the existence of several structures related to the one displayed in Fig. 5, very close in energy and differing only in minor conformational changes.

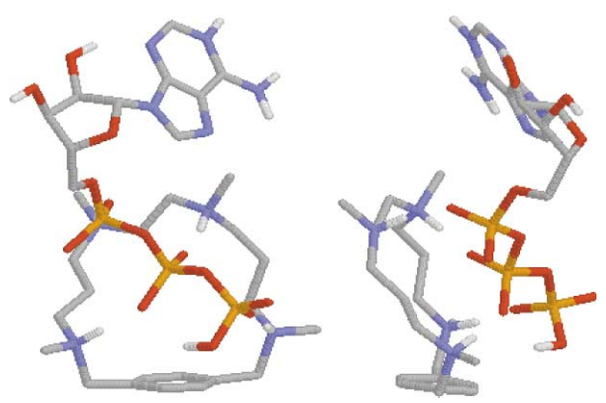


Fig. 5 Minimum energy conformer calculated for the interaction of tetraprotonated **B323Me$_4$** with ATP.

Conclusions

The present results clearly show how very minor structural changes can have a large influence in defining the nature and the strength of the interaction of protonated polynitrogenated receptors with anions of biological relevance, in this case ATP. In the present work, it has been observed that *N*-methylation of **B323** (**1**) is a very favourable feature when the interaction with ATP is considered. The resulting receptor, **B323Me$_4$** (**2**), is very selective for ATP over other phosphate anions such as ADP, AMP, PYR or TPP, with conditional constants, measured in water, being one to two orders of magnitude larger for ATP. When **B323Me$_4$** is compared with **B323**, it can be seen that *N*-methylation is accompanied by an increase in the corresponding conditional constants of at least two orders of magnitude over the entire range of pH. These results can be related with the observations reported by different authors regarding the improvement in DNA–receptor interaction with the use of polynitrogenated macrocycles containing alkyl chains.[16] Apparently, the increase in hydrophobicity can play a significant role. The lower ligand solvation is likely to contribute to the higher affinity constants observed as a consequence of the energetically less expensive desolvation.

Experimental

General synthetic procedure for the preparation of permethylated polyaza[*n*]paracyclophanes.

Synthesis of *N*,*N*′,*N*″,*N*‴-tetramethyl-2,6,9,13-tetraaza[14]-paracyclophane (B323Me$_4$, 2)

Compound **1** (0.13 g, 0.48 mmol) was dissolved in a mixture of formic acid (0.78 g, 13.55 mmol), formaldehyde (0.57 g, 6.19 mmol) and water (0.05 mL). The resulting solution was stirred and heated under reflux for 48 h and then cooled in an ice bath. The solution was then basified with NaOH until pH > 12 and extracted with CH_2Cl_2 (3 × 30 mL). The organic phase was dried and the solvent vacuum evaporated at reduced pressure to give an oily product (0.158 g, 99%). ^{1}H NMR ($CDCl_3$, δ ppm): 1.28 (m, 4H), 1.96 (s, 6H), 1.97–2.22 (m, 12H), 2.26 (s, 6H), 3.40 (s, 4H), 7.24 (s, 4H). ^{13}C NMR ($CDCl_3$, δ ppm): 26.6, 45.8, 46.9, 54.9, 56.3, 58.4, 65.2, 139.2, 140.4. MS (ESI)-(M + H): 333. This compound was stored as its hydrobromide and the elemental analysis was obtained from the corresponding salt. Calc. for $C_{20}H_{36}N_4$·4HBr·$H_2$0: C, 36.6; H, 6.1; N, 8.5. Found: C, 36.6; H, 6.3; N, 8.6%.

N,*N*′,*N*″,*N*‴-Tetramethyl-2,5,8,11-tetraaza[12]paracyclophane (B222Me$_4$, 4)

99% Yield. ^{1}H NMR ($CDCl_3$, δ ppm): 1.89 (s, 4H), 2.02 (s, 6H), 2.00–2.25 (m, 8H), 2.27 (s, 6H), 3.46 (s, 4H), 7.29 (s, 4H). ^{13}C NMR ($CDCl_3$, δ ppm): 45.6, 47.7, 54.2, 55.2, 57.1, 65.2, 133.6, 140.7. MS (ESI)(M + H): 305. This compound was stored as a hydrobromide and the elemental analysis was obtained from the corresponding salt. Calc. for $C_{18}H_{32}N_4$·4HBr·5$H_2$0: C, 34.4; H, 5.7; N, 8.9. Found: C, 34.3; H, 5.9; N, 9.0%.

N,*N*′,*N*″,*N*‴-Tetramethyl-16,17,19,20-tetramethyl-2,5,8,11-tetraaza[12]paracyclophane (D222Me$_4$, 6)

99% Yield. ^{1}H NMR ($CDCl_3$, δ ppm): 1.96 (s, 4H), 2.05 (m, 4H), 2.12–2.24 (m + s, 10H), 2.31 (s, 12H), 2.39 (s, 6H), 3.62 (s, 4H). ^{13}C NMR ($CDCl_3$, δ ppm): 16.8, 44.1, 44.7, 51.4, 52.3, 55.9, 56.8, 134.0, 134.6. MS (ESI)(M + H): 361. Elemental analyses always gave inconsistent results with this compound.

Emf measurements

The potentiometric titrations were carried out at 298.1 ± 0.1 K using 0.15 mol dm^{-3} NaCl as a supporting electrolyte. The

experimental procedure (burette, potentiometer, cell, stirrer, microcomputer, *etc*.) has been fully described elsewhere.[7] The acquisition of the emf data was performed with the computer program PASAT.[8] The reference electrode was a Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen-ion concentration probe by titration of previously standarised amounts of HCl with CO_2-free NaOH solutions and the equivalent point was determined by the Gran method,[17] which gives the standard potential, $E^{\circ\prime}$, and the ionic product of water ($pK_w = 13.73(1)$).

The computer program HYPERQUAD was used to calculate the protonation and stability constants.[9] The pH range investigated was 2.5–10.5. The different titration curves for each system (at least two) were treated either as a single set or as separated curves without significant variations in the values of the stability constants. Finally, the sets of data were merged together and treated simultaneously to give the final stability constants.

NMR measurements

The ^{1}H, ^{13}C and ^{31}P spectra were recorded on VARIAN MERCURY 300 and VARIAN INOVA 500 spectrometers, operating at 300 and 500 MHz for ^{1}H, 75.43 and 125.75 MHz for ^{13}C, and 121.44 and 202.40 MHz for ^{31}P. The ^{15}N spectra were measured on a VARIAN INOVA 500 spectrometer equipped with a 5 mm, tunable, broadband, inverse-detection probe at 50.7 MHz.[12]

Molecular mechanics calculations

Calculations were carried out on a Silicon Graphics workstation using the package of programs implemented in MACROMODEL 5.0.[15] Conformational searches were made using the Monte-Carlo method in the automatic mode and with standard parameters and cut-offs. At least three conformational searches were used for each case under study using 1000 structures. Solvents were simulated using the continuous method GB/SA as implemented in MACROMODEL.

Acknowledgements

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