Molecular Recognition of Nucleotide Pairs by a Cyclo-Bis-Intercaland-Type Receptor Molecule: A Spectrophotometric and Electrospray Mass Spectrometry Study

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Abstract: The water-soluble cyclo-bis-intercaland-type receptor molecules **3a–c**, positively charged macrocyclic polyamines containing two large crescent-shaped quinacridine subunits, bind anionic aromatic substrates, such as nucleotides, by a combination of π-stacking and electrostatic noncovalent interactions. Absorption and fluorimetric experiments in aqueous solution show that **3a–c** form 1:2 (host/guest) noncovalent complexes with nucleoside monophosphates and 1:1 complexes with nucleoside di- and triphosphates. Higher affinities are found for guanosine derivatives than for other nucleobases. This unusual binding of a nucleoside monophosphate pair involves π-stacking/hydrophobic effects between the nucleobases and the quinacridine subunits of hosts **3a–c** and possible hydrogen bonding between the two nucleobases within the complex. The results are compared with those obtained with acyclic monomer molecules **4a–b** and with analogous cyclo-bis-intercalands **1** and **2** bearing smaller aromatic subunits. An electrospray ionization mass spectrometry (ESI-MS) analysis of the noncovalent associations formed between **3a** and nucleoside monophosphates is presented. This technique based on the determination of molecular weight allows the observation of the major 1:2 complexes present in solution with a good preservation of the noncovalent associations during the ionization process. Competition experiments show that the binding selectivity observed for the guanosine derivatives in aqueous solution is preserved in the gas phase.

Keywords: bis-intercalands · host–guest chemistry · mass spectrometry · molecular recognition · nucleotides

Introduction

The study of the molecular recognition of nucleobases and their derivatives by synthetic hosts is aimed at a better understanding of the noncovalent interactions that play a role in relevant biological processes and may also contribute to the design of artificial receptors for therapeutic purposes.

In the past years, the selective binding of nucleobase derivatives has received much attention, but only few studies have been devoted to water-soluble host molecules, which have a better biomimetic scope.[1] Most of them are polytopic receptors, which are able to anchor nucleotides through several sites by using one or several types of weak interactions, that is, electrostatics, π-stacking, H-bonding, and hydrophobic effects. In particular, polyamino molecules, which are protonated in aqueous media around the physiological pH, have been widely used for their ability to interact strongly with the negatively charged phosphate groups of nucleotides. Natural polyamines such as spermine and spermidine,[2] as well as macrocyclic polyamines,[3–5] have indeed proven to be efficient and even selective tools for the binding of nucleotides, with affinity constants up to $10^{11}\,M^{-1}$.[3d] Macrocyclic polyamines bearing aromatic heterocyclic residues[3f,g] and polyamino derivatives of cyclophanes and cyclodextrins have also demonstrated their ability to bind nucleobase derivatives in water, owing to a combination of electrostatic and stacking interactions together with hydrophobic effects.[6]

The molecular recognition of nucleotides by rigid cyclo-bis-intercaland-type cyclophanes has been reported.[7] These compounds are macrocyclic molecules composed of two aromatic subunits linked by two spacers and bind aromatic substrates by using both electrostatic interactions and hydro-

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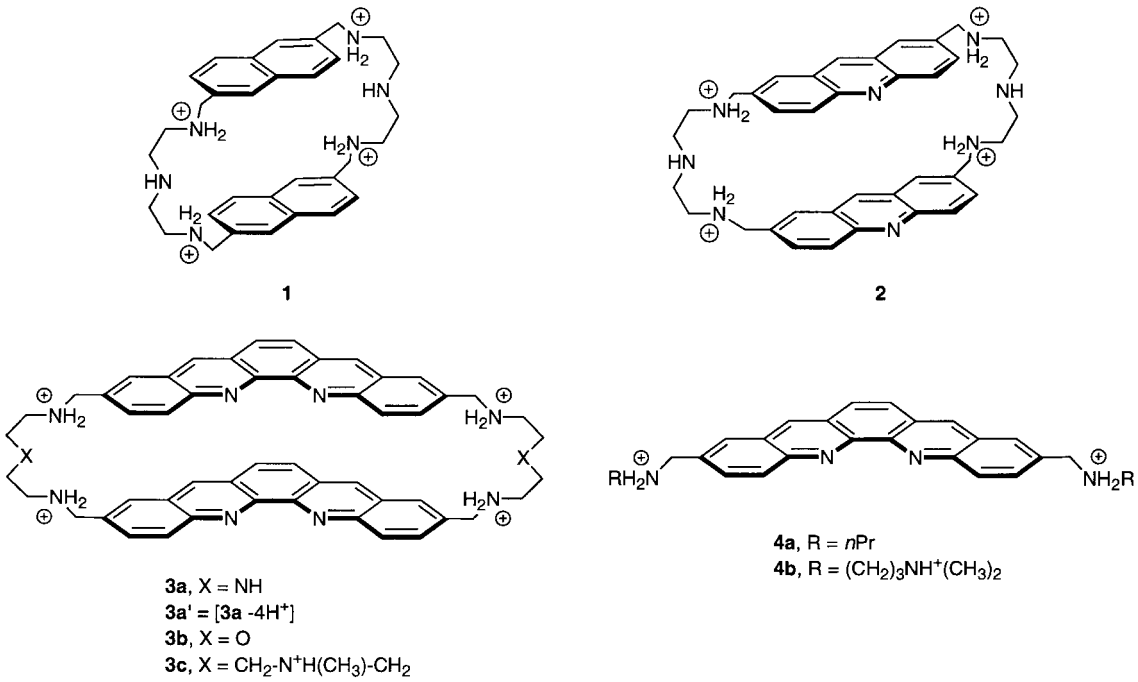

Figure 1. Schematic representation of macrocyclic compounds **1**, **2**, and **3a–c** and of monomeric compounds **4a–b** in their major protonated form at pH 6.

Abstract in French: *Les récepteurs hydrosolubles de type cyclo-bisintercalant **3a–c**, polyamines macrocycliques positivement chargées contenant deux sous-unités quinacridine de grande surface et de forme coudée, s'associent à des substrats anioniques aromatiques tels que les nucléotides par une combinaison d'interactions électrostatiques et de π-stacking. Des expériences de spectroscopie d'absorption et de fluorescence en solution aqueuse montrent que **3a–c** forment des complexes non covalents de st + chiométrie 1:2 (récepteur/substrat) avec les nucléosides monophosphate et des complexes de st + chiométrie 1:1 avec les nucléosides di- et triphosphate. Des affinités plus élevées sont observées pour les dérivés de la guanosine par rapport aux autres bases nucléiques. Cette complexation originale d'une paire de nucléosides monophosphate met en jeu des effets hydrophobes et de π-stacking entre les bases nucléiques et les sous-unités quinacridine des récepteurs **3a–c** et pourrait également faire intervenir des liaisons hydrogène entre les deux bases à l'intérieur du complexe. Les résultats sont comparés avec ceux obtenus avec les molécules monomères acycliques **4a–b** et avec les cyclobisintercalants analogues **1** et **2** qui portent des sous-unités aromatiques de plus petite taille. Une analyse par spectrométrie de masse à ionisation électrospray (ESI-MS) des associations non covalentes form´es entre **3a** et les nucléosides monophosphate est présentée. Cette technique basée sur la détermination de la masse moléculaire permet d'observer les complexes 1:2 majoritaires présents en solution avec une bonne conservation des associations non covalentes pendant le processus de désorption-ionisation. Des expériences de compétition montrent que la sélectivité de complexation observée pour les dérivés de la guanosine en solution aqueuse est conservée en phase gazeuse.*

phobic/π-stacking effects. High affinity constants were measured ($10^4-10^6\,M^{-1}$) but with almost no selectivity for a particular nucleobase or nucleotide charge; this was ascribed to the rigidity of the host molecules. Significant improvements were made with the design of the flexible bis-naphtalene **1** and bis-acridine **2** cyclo-bis-intercaland molecules (Figure 1).[8] These macrocycles were found to strongly bind nucleotides ($10^4-10^8\,M^{-1}$), with stability constants increasing with the charge of the substrate ($AMP^{2-} < ADP^{3-} < ATP^{4-}$). Moreover, **2** shows a binding selectivity toward purines and especially adenosine derivatives, with stability constants up to $10^8\,M^{-1}$ for ATP^{4-}. The increased affinity for nucleoside di- and triphosphates relative to monophosphates may be attributed to the strong electrostatic interactions displayed by the tetraprotonated macrocyclic receptors towards the multiply charged anionic substrates and demonstrates the role of the polyammonium subunits of these host molecules. The selectivity toward purine derivatives observed with **2** compared with **1** was ascribed to the extended aromatic surface of the acridine moiety, which entails a higher contribution of the π-stacking interactions with aromatic substrates; thus, nucleobases possessing a larger aromatic surface, that is, purines, bind to receptor **2** more tightly than pyrimidines. Recent crystallographic data have shown that, with flat aromatic carboxylates, **1** and **2** form inclusion complexes in which the substrate is inserted between the planar intercalator groups of the receptor.[9] Such structures have not been structurally characterized so far with **1** or **2** and nucleotides although they have been suggested by spectroscopic methods.[8]

Nucleobases do not significantly associate in dilute aqueous solutions through hydrogen bonding owing to the weakness of this interaction in water.[10] This association could nevertheless be favored upon complexation by host molecules containing a local hydrophobic microenvironment in which

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hydrogen bonding should be enhanced. Such a process could mimick the base-pairing phenomena that occurs in biological systems. Nucleobase pairing in water has been studied by complexation to a polytopic receptor, to which one of the two complementary bases was directly attached in a covalent manner.[11–12] The cyclo-bis-intercaland molecule **3a** was designed as a potential receptor for promoting the formation of pairs between two free nucleobase derivatives.[13] This compound contains the same polyamino bridges as **1** and **2**, but with larger aromatic subunits of the quinacridine (dibenzo[*b*,*j*][1,10]phenanthroline) type. The shape and size of these pentacyclic heterocycles are such that these units may overlap almost completely with a nucleobase pair associated through the Watson–Crick mode.[13] Bis-quinacridine receptor **3a** might therefore display appropriate interactional (in particular strong π-stacking) and steric complementarities with nucleotide pairs and thus favor their association in aqueous solution.

In the present study we describe the determination of the stoichiometry and stability constants of the noncovalent complexes formed between the cyclo-bis-intercaland molecule **3a** and nucleotides in aqueous solution by spectrophotometric methods. For comparative purposes the binding properties of macrocycles **3b** and **3c**, which bear different polyamine chains, and mono-quinacridines **4a** and **4b** were also investigated (Figure 1). In addition we report a study by electrospray ionization mass spectrometry (ESI-MS), aimed at providing a direct characterization of the stoichiometry of nucleotide complexes by their molecular weight related to gas-phase stabilities. Since the introduction in 1988 of ESI-MS as a tool for the study of large biomolecules,[14] this technique, as well as others such as FAB and MALDI, has been used successfully to probe noncovalent associations[15] and for the detection of the complexes of ligands with proteins[16,17] and DNA[18], of inclusion complexes[19] for instance with cyclodextrins,[20] as well as for the investigation of self-assembling systems.[21] Therefore ESI-MS could be a suitable method to study the complexes formed between cyclo-bis-intercaland host molecules and nucleotides, and might provide important information about the behavior of these complexes in aqueous solution. In order to perform a successful MS analysis of the preformed noncovalent complexes, the following criteria must be satisfied: i) the ESI source must be capable of desorbing intact complex ions in the gas phase from the solution; ii) the internal energy transferred to the ions during the desolvation process must be minimal, to prevent their dissociation; iii) the sequential MS/MS method must allow the fragmentation of the complexes in order to compare their stability in the gas phase; this by extension may be likely to yield information on their stability in the liquid phase.

Results and Discussion

Spectroscopic studies in water with 3a: The high molar-extinction coefficients and quantum yields of receptors **3** prompted us to monitor the complexation of nucleotides by these molecules using spectrophotometric titrations.[13] The great sensitivity of these methods generally allows the use of dilute conditions and affords accurate results. All studies were conducted at pH 6.0, in a PIPES buffer. At this pH, ribonucleotides are expected to be predominantly in their fully ionized form: cXMP$^-$, XMP^{2-}, XDP^{3-}, XTP^{4-}(c = cyclic phosphate; X = A, G, U, C). For the fluorescence measurements, the excitation wavelength was fixed at the isobestic point appearing in the absorption spectrum of **3a–c** during the titration, that is, 360 nm, at which the absorbance of all nucleotides is negligible, so that there was no variation in the excitation energy during the experiment. The addition of gradual quantities of nucleotides induced a progressive decrease of the absorption and fluorescence spectra of macrocycle **3a** (a typical experiment is shown in Figure 2A). The total hypochromism and fluorescence quenching reached

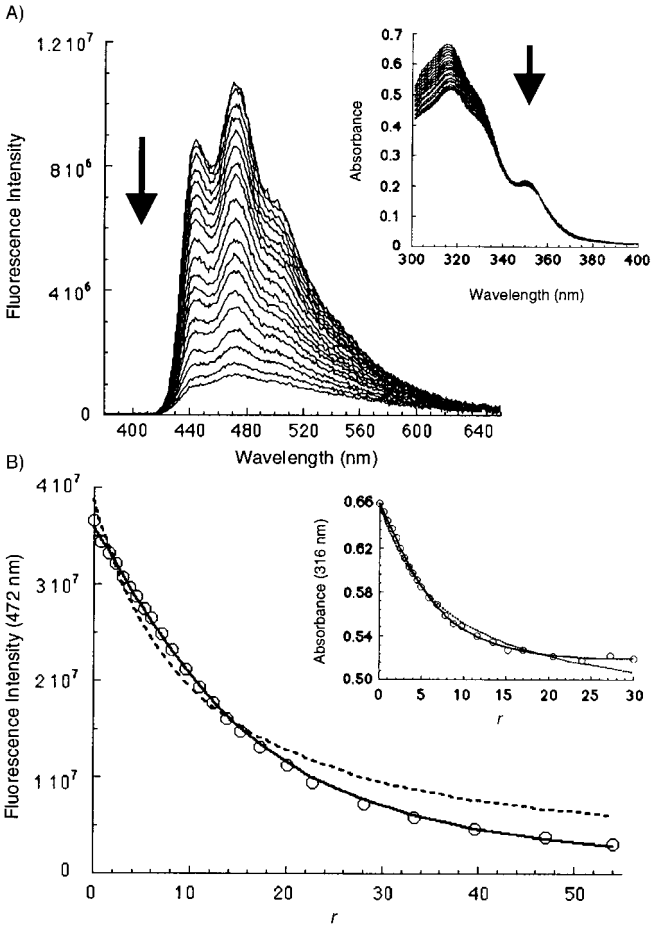

Figure 2. Fluorimetric and (inset) absorption titration of **3a** with GMP^{2-} at pH 6.0 (1 mM PIPES buffer): A) fluorescence and (inset) absorption spectra of **3a** (λ_{exc} = 360 nm) recorded with increasing concentration of substrate; B) experimental (circles) and calculated titration curves obtained for a 1:1 (dashed line) or a 1:2 (solid line) stoichiometry, shown at a fluorescence intensity of 472 nm and (inset) an absorbance of 316 nm (r = [GMP^{2-}]/[**3a**]).

15–60% and 25–95%, respectively (Table 1). It is well known that upon binding to acridines nucleobases induce a modification of the fluorescence of these dyes, with a quenching or an enhancement that depends on the acridine derivative. [22] This effect is commonly ascribed to an overlap

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Table 1. Association constants ($\log K_i$), free energies of complexation (ΔG_i°), fluorescence quenching ($\Delta\Phi/\Phi_0$), and hypochromicity ($\Delta A/A_0$) calculated for the complexes formed between receptor **3a** and various nucleotides.

Nucleotide[a]	$\log K_1$[b]	$\log K_2$[b]	$-\Delta G_1^\circ$[c] [kJ mol^{-1}]	$-\Delta G_2^\circ$[c] [kJ mol^{-1}]	$\Delta\Phi/\Phi_0$[d]	$\Delta A/A_0$[e]
G, A, C, U	<2	–[f]	<11	–	<0.05	<0.05
3′,5′-cGMP$^-$	3.1	3.6	17.4	20.2	0.95	0.2
2′,3′-cGMP$^-$	2.9	3.7	16.3	20.7	0.95	0.2
GMP^{2-}	4.1	4.5	23.0	25.2	0.95	0.25
AMP^{2-}	4.2	3.2	23.5	17.9	0.45	0.15
CMP^{2-}	3.8	3.3	21.3	18.5	0.5	0.15
UMP^{2-}	<3	–[f]	<17	–	0.25	0.15
GDP^{3-}	5.2		29.2		0.95	0.35
ADP^{3-}	4.4		24.7		0.45	0.25
GTP^{4-}	6.8		38.1		0.95	0.6
ATP^{4-}	5.4		30.3		0.65	0.5
UTP^{4-}	4.4		24.7		0.4	0.45

[a] G: guanosine A: adenosine,C: cytidine, U: uridine; 3′,5′-cGMP$^-$ and 2′,3′-cGMP$^-$ are 3′,5′- and 2′,3′-cyclic guanosine monophosphate, respectively. [b] Measured by fluorimetric or absorption titrations (20°C), estimated precision ± 0.2; K_1 and K_2 refer to the respective equilibria $M+N \rightleftharpoons MN$ and $MN+N \rightleftharpoons MN_2$ (M = macrocyclic receptor **3a**, N = nucleotide). [c] Calculated from the association constants. [d] $\Delta\Phi = \Phi_0 - \Phi$ difference between the areas of the emission peaks of the receptor **3a** free (Φ_0) and bound to the substrate (Φ). [e] $\Delta A = A_0 - A$ difference between the absorbances of receptor **3a** free (A_0) and bound to the substrate (A). [f] Not measurable.

between the acridine and the nucleobase rings indicating the formation of a molecular complex. By analogy, the fluorescence quenching observed upon binding of all nucleotides to **3a**, which is especially strong with guanosine derivatives, may reflect the presence of $\pi-\pi$ interactions between the nucleobases and the quinacridine subunits of **3a**. This quenching is likely ascribable to a less fluorescent steady-state complex between the two molecules, as suggested by the hypochromism in the UV spectra, rather than to a pure excited-state species.

The titration experiments have been analyzed by a nonlinear least-square curve-fitting program.[23] In the case of guanosine monophosphate derivatives cGMP$^-$ and GMP^{2-}, a non ambiguous fit was found for a 1:2 stoichiometry (**3a**/nucleotide), as shown in Figure 2B. In this case, the calculated curve for the 1:2 stoichiometry (solid line) matched almost perfectly the experimental points (circles), while the curve calculated for other stoichiometries, as for instance 1:1 (dashed line), did not. With all nucleotides other than GMP derivatives, both fitting curves for 1:2 and 1:1 stoichiometries were found to match the experimental points. Figure 3 shows the fluorimetric titration of **3a** by AMP^{2-} illustrating this behavior.

Among the methods that provide a reliable determination of the stoichiometry of noncovalent complexes, continuous variation plots known as the Job plots were used to characterize more thoroughly the species formed with **3a** and nucleotides.[24] For this purpose $\Delta A_f = A_f$ (free) $- A_f$ (bound) was plotted against the ratio of concentrations $R = [\mathbf{3a}]/([\mathbf{3a}] + [\text{nucleotide}])$, A_f being the area of the fluorescence emission peaks of **3a** free or bound to the nucleotide, at a constant total ($[\mathbf{3a}] + [\text{nucleotide}]$) concentration. Figure 4 shows the Job plots obtained for **3a** in the presence of GMP^{2-}

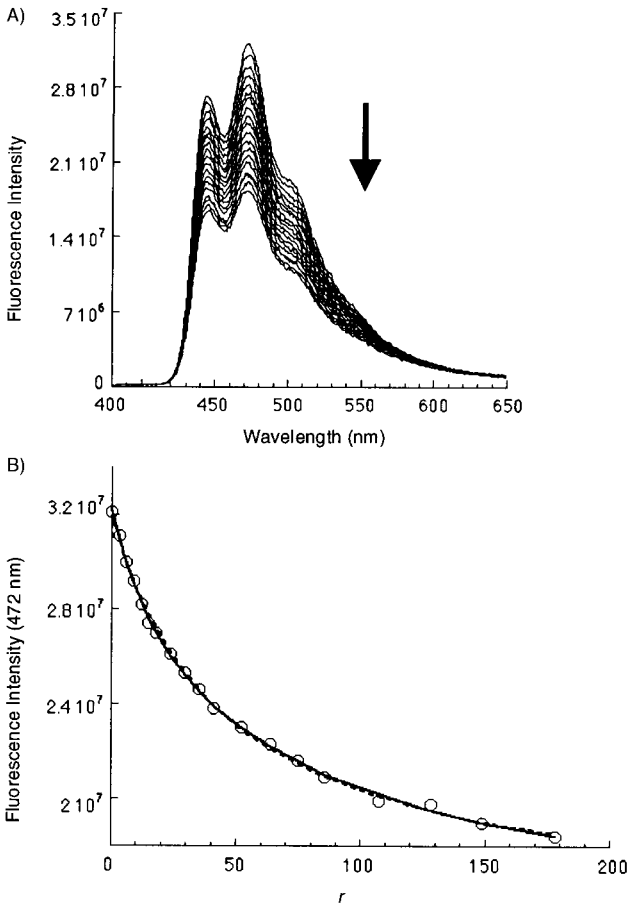

Figure 3. Fluorimetric titration of **3a** with AMP^{2-} at pH 6.0 (10 mM PIPES buffer): A) fluorescence spectra of **3a** ($\lambda_{exc} = 360$ nm) recorded with increasing concentration of substrate; B) experimental (circles) and calculated titration curves obtained for a 1:1 (dashed line) or a 1:2 (solid line) stoichiometry, shown at a fluorescence intensity of 472 nm ($r = [\text{AMP}^{2-}]/[\mathbf{3a}]$).

or GTP^{4-}. The stoichiometry of the complex formed can be deduced from these curves at the abscissa of the maximum or at the abscissa of the intersection of the two tangents to the curves at $R = 0$ and $R = 1$. A maximum can be indeed observed at $R = 0.37$ for GMP^{2-} (Figure 4A), indicating that a 1:2 complex is formed rather than a 1:1 complex.[24] With GTP^{4-} (Figure 4B), the Job method gave a maximum at 0.5, indicative of a 1:1 stoichiometry. The stoichiometry and stability constants of each complex with **3a** and nucleotides can therefore be determined accurately from both titration and Job plots experiments. As can be observed in Table 1 all nucleoside monophosphates, including cGMP$^-$ which bears a single negative charge at pH 6.0, form 1:2 complexes with **3a**, whereas nucleoside diphosphates and triphosphates give 1:1 complexes. Among nucleoside monophosphates bearing the same charge, the sequence of overall binding constants ($K = K_1 \times K_2$) is $\text{GMP}^{2-} > \text{AMP}^{2-} > \text{CMP}^{2-} > \text{UMP}^{2-}$. When the charge of the nucleotide increases (di- and triphosphate derivatives), the selectivity for the guanosine derivatives is preserved, with $K_1(\text{GDP}^{3-}) > K_1(\text{ADP}^{3-})$ and $K_1(\text{GTP}^{4-}) > K_1(\text{ATP}^{4-})$.

The comparison of the present results with those obtained with the former cyclo-bis-intercaland hosts **1** and **2** empha-

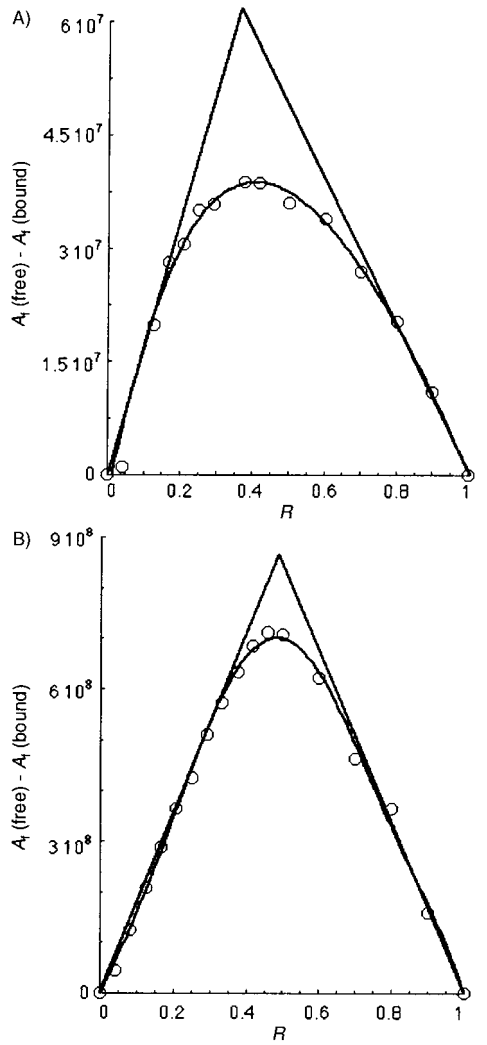

Figure 4. Job plots of mixtures of **3a** and A) GMP^{2-} or B) GTP^{4-}, recorded at pH 6.0 (1 mM PIPES buffer). A_f area of the emission peaks of **3a** free or in presence of the substrate ($\lambda_{exc} = 360$ nm); $R = [\mathbf{3a}]/([\mathbf{3a}] + [\text{nucleotide}])$; the total ($[\mathbf{3a}] + [\text{nucleotide}]$) concentration was 6×10^{-6} M.

sizes the role of the large crescent-shaped quinacridine rings. With **1** and **2** 1:1 complexes were formed with nucleotides, with greater stability constants and selectivities for the bisacridine **2**; both of these features are ascribable to the extended hydrophobic interactions displayed by the larger acridine ring.[8] When the area of the aromatic subunits of the cyclo-bis-intercaland host increases further, that is, with **3a**, the complexation of two molecules of substrate seems to be promoted instead. This is probably due to a good structural complementarity between the host and guest molecules compatible with both the anchorage of one nucleotide on each cationic bridge and the $\pi-\pi$ overlap between the quinacridine units. The two nucleobases can both fit inside the cavity. The values of K_1 measured for all nucleoside monophosphates are lower than those obtained with the bisacridine analogue **2**;[8b] this might indicate that the bisquinacridine structure is too large to accomodate one nucleotide with a good fit. Therefore the 1:2 stoichiometry observed with nucleoside monophosphates validates the choice of the quinacridine moiety to build cyclo-bis-intercaland host molecules capable of binding a pair of nucleotides. In the absence of more information about their exact geometry, only a schematic representation (Figure 5) of these 1:2 entities can be given and is warranted at this stage.

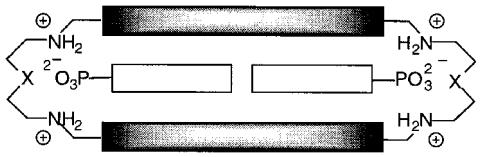

Figure 5. Schematic representation of the 1:2 complex structure formed between receptors **3a–b** and nucleoside monophosphates; (X = NH, O).

The differences in affinity of **3a** towards the nucleoside monophosphates (XMP^{2-}) and in particular the preferential complexation of purine derivatives is largely in favor of the significant contribution of hydrophobic effects to the stability. This result demonstrates unambiguously that the complexation is not purely driven by electrostatics despite the low affinity for the neutral analogues (G, A, C, U; Table 1). In addition, the selectivity observed for GMP^{2-} and the high affinity for the monocharged $cGMP^{-}$ derivatives, might also originate in stronger $\pi-\pi$ interactions dut to more favorable overlap of the quinacridine moieties with guanine pairs as compared with other nucleobase pairs. Another explanation could be the intervention of favorable guanine–guanine interactions within the complex, which will be discussed in the next section.

Finally, the observation of a 1:1 stoichiometry with di- and triphosphate derivatives might be explained by two factors related to the change in ionic and hydrophobic interactions within the receptor upon binding to the guest molecule. First, the binding of one molecule of the most charged nucleotides such as di- and triphosphate derivatives to **3a** is expected to produce a major decrease in the overall charge of the 1:1 complex and thus in the electrostatic attraction of a second molecule of substrate. Second, it is generally admitted that with cyclophane-type receptors the increase of the charge of the substrate induces a decrease of the hydrophobic interactions due to displacement of the lipophilic parts from ideal contact. [25] Such effects as well as steric compression, might disfavor the formation of a 1:2 complex to the benefit of a 1:1 association.

Spectroscopic studies in water with 3b,c and 4a,b: Stoichiometries and stability constants of the complexes formed between **3b,c** and nucleoside monophosphates have been investigated in the same manner as above for **3a** (Table 2). A

Table 2. Association constants ($\log K_i$) calculated for the complexes formed between receptors **3a–c** and nucleoside monophosphates.[a]

	$\log K_1$, $\log K_2$		
	3a	**3b**	**3c**
3′,5′-$cGMP^{-}$	3.1 , 3.6	3.0 , 3.3	< 3, –[b]
GMP^{2-}	4.1 , 4.5	3.4 , 5.0	3.7 , 4.3
AMP^{2-}	4.2 , 3.2	4.4 , 3.0	3.2 , 2.6
CMP^{2-}	3.8 , 3.3	3.9 , 2.6	< 3, –[b]
UMP^{2-}	< 3, –[b]	< 3, –[b]	< 3, –[b]

[a] See footnote to Table 1. [b] Stoichiometry not measurable.

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1:2 stoichiometry was observed, as with **3a**, in all cases where stability constants were high enough to allow a mathematical treatment. A major trend is evident from the data : **3a** and **3b** have similar binding behaviors, whereas a decrease of the binding ability is clearly observed with **3c**. The structure of macrocycle **3b** differs from that of **3a** only by the nature of the central heteroatom of the side chains and thus the close values of the stability constants measured with both macrocycles are in accordance with expectations. On the other hand, larger binding constants could have been anticipated with **3c**, which possesses an additional positive charge on each central nitrogen atom,[13] thus favoring stronger ionic interactions with the phosphate groups. The propylene triamine linkers, however, serve not only to increase the basicity of the central nitrogen atom, but also to significantly enhance the interchromophoric distance relative to that of **3a**.[13] Thus the effect of the increase of the electrostatics due to the higher cationic charge of the receptor seems to be counterbalanced by the influence of the larger distance between the two quinacridine units, which should reasonably diminish the stacking effect. The decrease of the overall stability constant ($K = K_1 \times K_2$) recorded with the purine derivatives implies that the hydrophobic and π-stacking effects contribute significantly to the noncovalent interactions in the complexes with nucleotide monophosphates, in line with the results obtained with **3a** and similar systems.[6–8, 25] These results, as well as the weak binding of $cGMP^-$ and CMP^{2-}, suggest that the reduced affinity might be due to a less tight fit of the nucleotides in the intramolecular cavity of receptor **3c**.

The importance of the „sandwichlike“ effect (i.e., stacking of the guest in between the two aromatic units of the host) is furthermore confirmed by complexation experiments with the reference monomers **4**. Indeed **4a** and especially **4b**, which bears four positive charges like **3a**, form much less stable complexes with nucleotides; for instance a stability constant of approximately $1000\,M^{-1}$ was estimated from spectroscopic titrations of the two derivatives with GMP^{2-}, but these low values did not permit Job plots to be performed to allow discrimination between 1:1 and 1:2 stoichiometry. However, this demonstrates the importance of the bis-quinacridine structure on the strength of the association with nucleotides and suggests that an inclusion complex is formed with macrocycles **3** in a semiclosed conformation, with the nucleobase pair stacked between the quinacridine units, similar to the crystal structures of macrocycles **1** and **2** with aromatic anions.[9] A more detailed analysis of the geometry of these complexes by NMR spectroscopy was unfortunately precluded by precipitation and signal broadening.

Finally a particular and important feature of the complexes with guanosine appears from the data of Table 2: the binding of the second nucleotide molecule is easier than that of the first ($K_2 > K_1$), which is the case with all three receptors (**3a–c**). The inverse ($K_2 < K_1$) is observed for all the other nucleotides. The facilitated anchorage of the second GMP^{2-} can result from specific GMP^{2-}/GMP^{2-} interactions (cooperative binding) within the complex or from the conformational change of the macrocycle induced by the binding of the first molecule of nucleotide (induced fit or allosteric effect). A reasonable explanation for cooperative binding would be the intervention of specific hydrogen bonding between the two guanines as a consequence of the proximity of the two bases, promoted by the hydrophobicity of the environment. Indeed guanines are well known to form stable nonclassical homobase pairings,[26] which leads for instance to the formation of tetrads,[27] and this occurs to a larger extent than with other nucleobases. If this is the case, experiments with mixtures of Watson–Crick complementary bases could be very informative about the formation of hydrogen bonding inside the complexes. Fluorimetric and UV/visible titrations with mixtures of GMP^{2-} and CMP^{2-} were conducted; however, they did not give evidence for the formation of a ternary complex (**3a** · GMP^{2-} · CMP^{2-}). On the contrary, CMP^{2-} seems to be displaced from **3a** upon addition of GMP^{2-} with formation of **3a** · $(GMP^{2-})_2$; as the area of a homopurine association such as G–G is larger than that of a Watson–Crick hydrogen-bonded pair such as G–C, these results could again indicate that π-stacking contacts make a major contribution in these complexes.

The preceding spectrophotometric experiments showed that noncovalent complexes are formed with one molecule of bis-quinacridine **3a–c** and two identical molecules of any nucleotide monophosphate in aqueous solution. However, the selectivity for guanines derivatives raised some interesting questions, particularly with respect to the formation of hydrogen bonds inside the complex. Thus, in order to gain more information about the complexes formed between receptors **3** and complementary bases and to extend the range of the solution studies, investigations were initiated towards the analysis of mixtures of **3a** and nucleotides by electrospray ionization mass spectrometry (ESI-MS).

Analysis of the binding of 3a to nucleoside monophosphates by ion trap ESI-MS: The binding of nucleoside monophosphates XMP^{2-} (X = A, T, G, C) to **3a** was evaluated by two series of ESI-MS experiments. In a first experiment, mixtures of **3a** and a single nucleotide at a **3a**/XMP^{2-} concentration ratio of 1:2 were analyzed, and in a second experiment mixtures of **3a** and two different nucleotides in 1:1:1 ratio were investigated. A step-by-step procedure was followed in order to find optimal conditions to observe specific complexes, that is, the species formed in the infused aqueous solution prior to desorption–ionization. The solutions were prepared in such a way as to get as close as possible to the experimental conditions used in the UV/visible and fluorescence studies. Since it is known that sodium adducts highly complicate the ESI mass spectra both in positive and negative modes,[28] nucleoside monophosphates were purchased in their acidic form and dissolved in dilute aqueous ammonia. The **3a**/XMP^{2-} mixtures were maintained at pH 6.0 by addition of a triethylammonium acetate buffer, which is volatile enough to minimize nonspecific triethylammonium adducts with other species, such as cations formed in the gas phase by desolvation. A 2:1 water/methanol mixture was chosen as the solvent to favor stable spray formation. It was checked by fluorimetric titration that under these buffer and solvent conditions, the stoichiometry and association constants of **3a** with nucleoside monophosphates were similar to those previously determined. However, the concentrations

used in the fluorescence experiments (3 μM in **3a** with a 50–200-fold excess of nucleotide) were not transposable to the ESI studies, since the use of an excess of nucleotide gave ESI mass spectra displaying mostly multiple nonspecific associations of nucleotides, as already described for experiments in the positive mode.[29] Therefore, we operated at a higher macrocycle concentration (150 μM of **3a**), which allowed the formation of a sufficient amount of complex in the presence of only two equivalents of nucleotide, thus giving acceptable sensitivities with low proportions of nucleobase clusters.

Under our experimental conditions, only ions bearing a single positive charge were detected, corresponding to monoprotonated species. For proteins the survival of only one charge per 1000 Da has been commonly observed during ESI-MS analysis.[30] Moreover, in view of the very fast rate of proton exchange during desorption, a variation of the degree of protonation of the species may somewhat affect the relative quantities of charged species in the gas phase as compared with those in solution. Since the position of the proton cannot be specified, the molecular associations detected in these experiments may be considered as monoprotonated derivatives of complexes between neutral components: $[\mathbf{3a'}+XMP+H^+]^+$, $[\mathbf{3a'}+2XMP+H^+]^+$, $[\mathbf{3a'}+3XMP+H^+]^+$, in which **3a′** designates the parent unprotonated form corresponding to $[\mathbf{3a}-4H^+]$ and XMP the acidic form $[XMP^{2-}+2H^+]$.

The ESI mass spectra obtained from solutions containing 150 μM of **3a** and two equivalents of GMP^{2-} (Figure 6A) or AMP^{2-} (Figure 6B) were recorded under a high potential difference between the capillary exit voltage (200 V) and the skimmer voltage (5 V). The peak at *m*/*z* 816 corresponds to the quasi-molecular ion of **3a′**, that is, $[\mathbf{3a'}+H]^+$; the other ions appearing at higher *m*/*z* values correspond to XMP clusters (intensity ca. 5%) and to the 1:1 (*m*/*z* 1179), 1:2 (*m*/*z* 1542), and 1:3 (*m*/*z* 1906) complexes between **3a′** and nucleotides (intensities ca. 5, 16, and 5%, respectively; Table 3). It is generally accepted that there is a great influence of the variation of the difference between the capillary exit and the skimmer voltages, named declustering potential, on the mass spectra of noncovalent species.[31] Usually, noncovalent complexes are best observed under low declustering potentials and mild ion-source conditions.[19, 32] In the present case, the quasi-molecular ions corresponding to the 1:1 and especially 1:2 noncovalent complexes were also clearly observable at high capillary exit and low skimmer voltages, that is, under a high declustering potential. This quite unusual behavior has already been reported during the study of drug–DNA interactions.[18b] A lower declustering potential (capillary exit voltage = 150 V) produced more intense peaks characteristic of the hetero-complexes relative to the $[\mathbf{3a'}+H]^+$ peak, but gave also more background noise. The ESI mass spectra recorded with a high declustering potential (Figure 6) show that in both cases the 1:2 complex is the major ion. Besides the peaks corresponding to the specific 1:1 and 1:2 complexes, peaks characterizing nucleotide clusters and the higher order 1:3 complex can also be observed. Their formation might originate in the re-concentration of species that occurs during droplet evaporation and cluster ion desolvation; this could not be avoided completely, even by applying higher declustering potentials. On the basis of the relative intensities of the peaks and with respect to the experimental conditions, some remarks could be drawn concerning the stoichiometry. The intensity displayed by the species $[\mathbf{3a'}+3GMP+H]^+$ (5%) is similar to that of the XMP clusters $\{[2GMP+H]^+, [3GMP+H]^+\}$ (2–5%) and is weak relative to that of the 1:2 complex $[\mathbf{3a'}+2GMP+H]^+$ (16%); thus, the preponderance of the 2:1 complex seems to reflect the stoichiometry favored in solution, whereas the weak intensity of the 1:3 complex could be significant of a nonspecific association. Similar ESI mass spectra were obtained with other nucleoside monophosphates (CMP^{2-}, TMP^{2-}) and

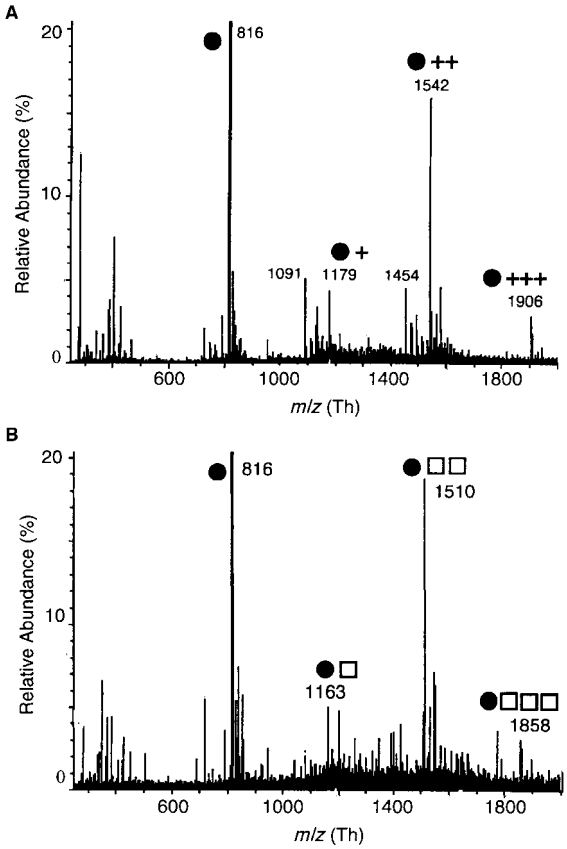

Figure 6. Electrospray mass spectra of mixtures of **3a** (1.5×10^{-4} M) and A) GMP^{2-} (2 equiv) or B) AMP^{2-} (2 equiv) recorded at a capillary voltage of 200 V and a skimmer voltage of 5 V. The quasi-molecular ion peak of **3a′** (*m*/*z* 816) is the base peak. Symbols of ions: ●, $[\mathbf{3a'}+H]^+$; ●+, $[\mathbf{3a'}+GMP+H]^+$; ●++, $[\mathbf{3a'}+2GMP+H]^+$; ●+++, $[\mathbf{3a'}+3GMP+H]^+$; ●□, $[\mathbf{3a'}+AMP+H]^+$; ●□□, $[\mathbf{3a'}+2AMP+H]^+$; ●□□□, $[\mathbf{3a'}+3AMP+H]^+$.

Table 3. The *m*/*z* values [Th] of singly charged ions observed in ESI mass spectra of mixtures containing receptor **3a′** and GMP, AMP or CMP.

	X = G	X = A	X = C
$[XMP+H]^+$	364	348	323
$[2XMP+H]^+$	727	695	647
$[\mathbf{3a'}+H]^+$	816	816	816
$[3XMP+H]^+$	1091	1043	971
$[\mathbf{3a'}+XMP+H]^+$	1179	1163	1139
$[4XMP+H]^+$	1454	1390	1294
$[\mathbf{3a'}+2XMP+H]^+$	1542	1510	1462
$[5XMP+H]^+$	1817	1737	1617
$[\mathbf{3a'}+3XMP+H]^+$	1906	1858	1786

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the relative intensities of the peaks corresponding to the 1:1 and 1:2 complexes turned out to be almost identical for all four nucleotides. This result is not in complete accordance with the relative affinities of **3a** for the various nucleotides provided from solution studies; thus, in an attempt to gain more information, competition experiments were conducted with equimolar mixtures of **3a** (150 μM) and two different nucleotides. This type of experiment could also allow investigations of the formation of complexes between **3a** and complementary base pairs. The positive-ion ESI mass spectrum of a **3a**/GMP^{2-}/AMP^{2-} solution mixture at a 1:1:1 ratio (Figure 7A), recorded with a high capillary exit voltage value

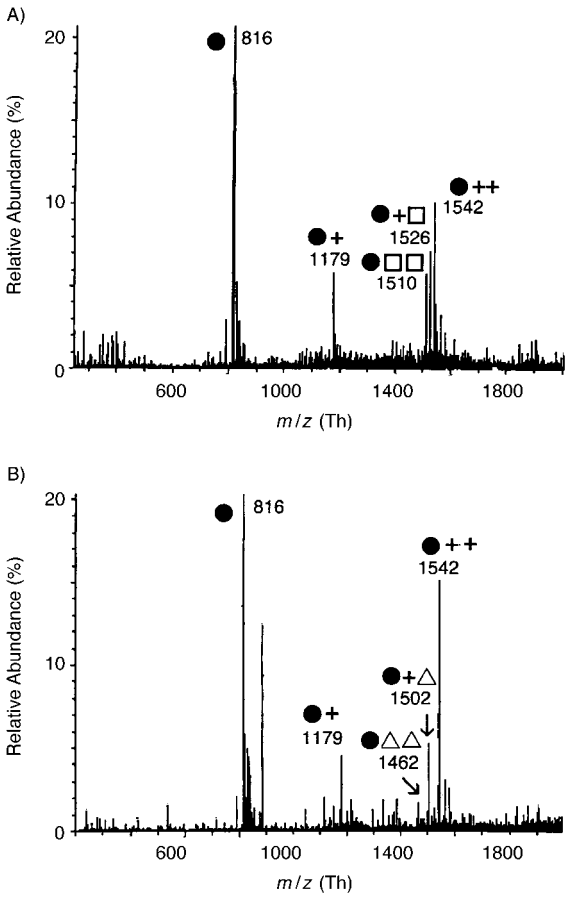

Figure 7. Electrospray mass spectra of mixtures of **3a** (1.5×10^{-4} M) and A) GMP^{2-} (1 equiv) and AMP^{2-} (1 equiv) and B) GMP^{2-} (1 equiv) and CMP^{2-} (1 equiv) recorded at a capillary voltage of 200 V and a skimmer voltage of 5 V. The quasi-molecular ion peak of **3a′** ($m/z = 816$) is the base peak. Symbols of ions: ●, $[\mathbf{3a'}+H]^+$; ●+, $[\mathbf{3a'}+GMP+H]^+$; ●△△, $[\mathbf{3a'}+2CMP+H]^+$; ●+△, $[\mathbf{3a'}+GMP+CMP+H]^+$; ●++, $[\mathbf{3a'}+2GMP+H]^+$; ●□□, $[\mathbf{3a'}+2AMP+H]^+$; ●+□, $[\mathbf{3a'}+GMP+AMP+H]^+$.

(200 V), allowed us to detect peaks corresponding to all mixed 1:2 complexes $[\mathbf{3a'}+2GMP+H]^+$, $[\mathbf{3a'}+GMP+AMP+H]^+$ and $[\mathbf{3a'}+2AMP+H]^+$ at m/z 1542, 1526 and 1510, respectively, with intensities in the following order: $[\mathbf{3a'}+2GMP+H]^+ > [\mathbf{3a'}+GMP+AMP+H]^+ > [\mathbf{3a'}+2AMP+H]^+$. In these declustering potential conditions, only one of the two 1:1 complexes is displayed, $[\mathbf{3a'}+GMP+H]^+$. It is noteworthy that only specific ions could be detected in this experiment. Similar trends can be observed from the analysis of the **3a**/GMP^{2-}/CMP^{2-} solution mixture (Figure 7B). Relative intensities are $[\mathbf{3a'}+2GMP+H]^+ > [\mathbf{3a'}+GMP+CMP+H]^+ > [\mathbf{3a'}+2CMP+H]^+$ and $[\mathbf{3a'}+GMP+H]^+ \gg [\mathbf{3a'}+CMP+H]^+$. When the capillary exit voltage is increased, the predominance of the $[\mathbf{3a'}+2GMP+H]^+$ complex peak is further enhanced over the others in both experiments. The 1:1 and 1:2 complexes formed with GMP^{2-} appear clearly to be the most stable associations towards dissociation in the gas phase. A selectivity for the 1:1 complex **3a′** · GMP appears in the gas phase in contrast to the solution where similar stabilities have been observed for complexes with GMP^{2-} and AMP^{2-} (Table 2). This selectivity was further confirmed by CID (collision-induced dissociation) of the $[\mathbf{3a'}+GMP+CMP+H]^+$ and $[\mathbf{3a'}+GMP+AMP+H]^+$ ions selected in the ion trap, which gave only the $[\mathbf{3a'}+GMP+H]^+$ and $[\mathbf{3a'}+H]^+$ daughter ions; each sequential MS/MS experiment was performed on the selected ion within the same conditions (data not shown).

In summary, the first set of experiments indicated the predominant formation of bis-quinacridine/nucleotide 1:2 complexes, which are also the major species in the aqueous phase. Electrospray ionization thus seems to offer a rapid and elegant method to evaluate the major noncovalent species present in solution. A major problem encountered in this study was the predominance of the quasi-molecular ion $[\mathbf{3a'}+H]^+$ over other ions; this originates from the low association constants of these complexes. Indeed, most studies in this field were performed with highly stable species (K_{ass} in the range $10^6 - 10^{12}$ M^{-1}),[17c, 33] which allowed a better conservation of noncovalent associations during the ionization–desorption process. The competition experiments confirm the selectivity for guanosine derivatives and moreover show that this selectivity is conserved for the 1:1 complex, which did not appear clearly from solution studies. The 1:2 complex formed with GMP^{2-} seems to be favored over any other 1:2 association and in particular it appears more stable than the 1:2 complex formed with complementary bases, that is, **3a′** · GMP · CMP; this might further indicate that hydrogen bonding does not play a major role in the complexation. The differences observed between behaviors in the gas phase and in the solution could be explained by the modification of noncovalent interactions, indeed hydrophobic effects are no longer present in the gas phase and interactions are mainly due to proton solvation related to the gas-phase basicities of each component.[34] Similar investigations with nucleoside di- and triphosphates, giving 1:1 complexes with bis-quinacridine **3a** are currently underway.

Conclusion

The cyclo-bis-intercaland-type host molecules **3a–c**, incorporating oligoammonium bridges linking two large crescent-shaped quinacridine subunits, have been shown to display noncovalent associations with nucleotides. In particular, the formation of 1:2 complexes containing two nucleoside monophosphates for one molecule of macrocyclic receptor was demonstrated, a recognition pattern which has not been described before with such host–guest systems. A binding

selectivity was observed with guanosine derivatives, which might originate in higher π-stacking interactions between the quinacridine moieties and this nucleobase. Hydrogen bonding between the two guanines is likely to occur in the hydrophobic microenvironment created by the cavity of the host molecules.

ESI-MS is a growing field for the analysis of noncovalent complexes, because it generally offers a reasonable preservation of noncovalent interactions in the gas phase, but it has been barely used for host–guest systems that possess low association constants. It was shown in this study that, under appropriate experimental conditions, it is possible to observe the major host–guest complex present in solution on a ESI-MS mass spectrum. This direct method based on the molecular weight determination seems to be particularly suited to confirm stoichiometries that were measured by more conventional solution studies. Further investigations are underway to gain structural information on the interaction of the two nucleotide molecules within the host compound.

Experimental Section

Materials: Compounds **3** and **4** were available from earlier work.[13] All nucleotides were purchased from Sigma.

Spectrophotometric titrations: UV/Vis experiments were monitored on a Beckman DU 460 spectrophotometer. Fluorescence measurements were performed on a Spex Fluoromax spectrophotometer equipped with a Hamamatsu R928 photomultiplier (PM); the data were corrected for the response of the PM. The nucleotides used in these experiments were ribonucleotide sodium salts. The buffer used was PIPES (1,4-piperazine-diethanesulfonic acid), pH 6.0, at a concentration of 1–10 mM depending on the total concentration of nucleotide added. The titrations were performed at a constant host molecule concentration of 3×10^{-6} M and 10^{-5} M for fluorescence and absorption experiments, respectively. The pH was checked at the beginning and at the end of the titration and was found to equal 6.0 in all cases. A temperature of 20°C was kept constant using thermostated cell holders. UV/Vis and fluorescence spectra were recorded 1 min after each addition in order to ensure that the equilibria were established (no change was observed with longer incubation time). Fluorimetric titrations were recorded at an excitation wavelength of 360 nm at which the absorption of the quinacridine compounds remained constant during the titration.

Job plots: Each sample was prepared in a 1 mM PIPES buffer (pH 6.0), at a constant $C = [M] + [N]$ concentration (M = macrocyclic receptor, N = nucleotide), in a total volume of 3×10^{-3} L. C was $6-10 \times 10^{-6}$ M, depending on the nucleotide. The ratio $[M]/C$ was fixed using calculated microliter portions of concentrated solutions of M and N. Two fluorescence spectra were recorded for each sample, in the same conditions as the titration experiments: one spectrum of the receptor alone at the concentration [M], giving fluorescence peak area A_f (free); one spectrum of the same sample after addition of the nucleotide at the concentration of $C - [M]$, giving peak area A_f (bound). The difference of the fluorescence peak areas A_f (free) $- A_f$ (bound) was plotted against the ratio $[M]/C$.

ESI mass spectrometry: All experiments were performed on an ESQUIRE ion trap (Bruker-Franzen Analytic GmbH, Bremen, Germany).[35] The nonlinear ion trap worked in the mass-selective instability mode without the use of DC voltage (i.e., $U = 0$ condition) at the ring electrode. The instrument has a fundamental RF frequency of 781 kHz and was used in the standard mode, with a mass-to-charge ratio up to 2000 Th [its scan used the nonlinear resonance at $\beta_z = 2/3$ ($q_z = 0.78$) for selective resonance ejection of ions with a 260 kHz frequency as axial modulation (analytical scan rate 8000 Th s^{-1})]. The ion trap operated at an uncorrected partial He buffer gas pressure of 3.4×10^{-5} Torr (4.5×10^{-3} Pa). A differentially pumped interface transferred the ions from the electrospray source (Analytica of Brandford, Brandford, CT) to the mass spectrometer. The N_2 counterflow was 400 L h^{-1} with a gas temperature of 200°C to allow desolvation of infused solutions. Some applied parameters were unchanged for all experiments, such as the RF frequency amplitude of the hexapole (350 V) and the voltage applied on the electron multiplier (−1500 V). In this positive-ion mode the DC voltage applied to the ion guide (hexapole) was 2 V, the dynode voltage of the electron multiplier −5 kV, the delay before scanning 5 ms, and the exit lens voltage −50 V. The low m/z cut-off used for the analytical scan of ions was chosen to put the ion at a q_z value of 0.048, in the aim to compare ion intensities (in this case ions have similar energies).

AMP^{2-}, GMP^{2-}, CMP^{2-}, and TMP^{2-} used in these experiments were purchased as free acids, which were dissolved at a concentration of 10^{-2} M in aqueous NH_4OH (10^{-2} M). Analyses were performed in the positive-ion mode. Solutions were freshly prepared in glass-free tubes and contained **3a** (1.5×10^{-4} M), one or two nucleotide equivalents, and triethylammonium acetate (3.5×10^{-3} M, pH 6.0) in H_2O/CH_3OH 2:1. They were infused into the ESI source by means of a Cole-Parmer 74900 Series syringe pump at a flow rate of 90 μL h^{-1}.

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