Multiple Molecular Recognition and Catalysis. A Multifunctional Anion Receptor Bearing an Anion Binding Site, an Intercalating Group, and a Catalytic Site for Nucleotide Binding and Hydrolysis†

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Abstract: The multifunctional receptor molecule **2** has been designed and synthesized in order to achieve higher molecular recognition and reaction selectivity via multiple interactions with bound substrates. It combines three functional subunits: two recognition sites—a macrocyclic polyammonium moiety as anion binding site and an acridine side-chain for stacking interactions—as well as a catalytic amino group in the macrocycle for facilitating hydrolytic reactions. Compound **2** binds mono- and dinucleotide polyphosphates by simultaneous interactions between its macrocyclic polycationic moiety and the polyphosphate chain as demonstrated by ^{31}P NMR spectroscopy and by stacking between its acridine derivative and the nucleic base of nucleotides as observed by both ^{1}H NMR spectroscopy and by fluorescence spectrophotometry. Binding of nucleotides by protonated **2** induces significant upfield shifts of the polyphosphate signals and of protons of the acridine moiety of **2** as well as of the adenine and the anomeric proton of the nucleotides; at the same time the proton signals corresponding to CH_2 groups of the macrocyclic part of **2** are downfield shifted. Upon complexation of ATP and CTP, the fluorescence emission of **2** is enhanced, whereas guanosine triphosphate causes a slight quenching; thus, **2** acts as a sensitive and selective fluorescent probe for ATP. At neutral pH the hydrolytic reaction proceeds, at least in part, through a covalent intermediate, the phosphorylated macrocycle **2** indicating nucleophilic catalysis. Compound **2** shows greater selectivity between ATP and ADP than the parent compound **1** which does not contain the acridine binding site. **2** also binds strongly to DNA plasmid pBR 322 at 10^{-6} M probably via a double type of interaction, involving both intercalation and electrostatic interactions with the phosphate groups.

Molecular recognition and supramolecular catalysis together with transport processes represent the basic functional features of supramolecular chemistry.[1-9] Molecular recognition of a substrate results from the readout of specific information concerning the substrate to be bound, which is stored at the molecular level within the structure of the receptor molecule. On the other hand, supramolecular catalysis involves, first, a binding step for which molecular recognition is a prerequisite, followed by the chemical transformation of the species bound within the supramolecular complex and finally the release of the products with regeneration of the catalytic unit.[5-9]

Anions play important roles in both chemical and biochemical processes,[10] and their complexation by synthetic macro(poly)cyclic polyammonium receptor molecules has been explored over the past 10 years.[7,9,11-23] These designed organic polycations form stable and selective complexes with a variety of inorganic as well as organic anions.[11-23] Among the biologically relevant anions, nucleotide polyphosphates, in particular adenosine mono-, and di-, and triphosphate are basic components in the bioenergetics of all living organisms,[24,25] the center for chemical energy storage and transfer being their polyphosphate chains. Macrocyclic polyamines, when protonated, bind strongly and selectively to nucleotides via electrostatic interactions between the cationic binding sites (ammonium groups) of the receptor and the negatively charged polyphosphate groups.[15a,16b,21-23] In order to attain better recognition of nucleotides, receptor molecules need to contain other binding sites, capable of interactions with the sugar moiety and/or the nucleic base, in addition to the anion binding sites. Interactions with the nucleic base may be achieved either by stacking, in which case distinction between different nucleic bases rests on differences in stacking energies, or by sites capable of forming complementary hydrogen bonding patterns, which should lead to molecular recognition between nucleic bases.[26-28] A combination of both may be needed if recognition is to take place in aqueous solution.

Since supramolecular catalysis involves initial substrate binding by the receptor molecule, followed by the transformation of the bound species, a better recognition may lead to a higher selectivity of reaction with different substrates. Nucleotide polyphosphates are particularly interesting in this regard since they undergo phosphoryl-transfer processes in both bond-cleavage as well as

† Dedicated to the memory of Professor E. T. Kaiser

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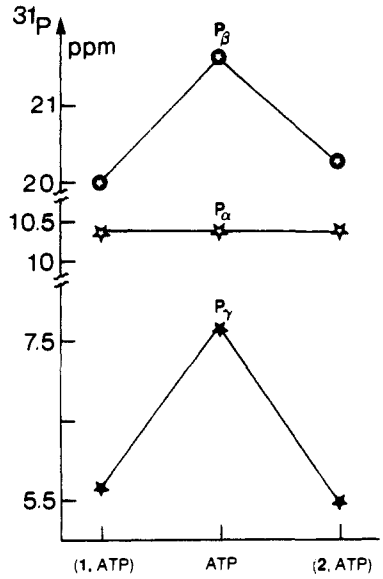

Figure 1. ^{31}P NMR chemical shifts of the α-, β-, and γ-phosphate signals of ATP in the absence and in the presence of equimolar amounts of macrocycles **1** and **2** at pH 7, in $D_2O/H_2O(1/9)$ solution.

in bond-formation reactions.[24,25] It has been shown previously that these phosphoryl-transfer reactions may be catalyzed by macrocyclic polyamines.[29-34] We now report the synthesis and a detailed study of a multifunctional receptor molecule **2** containing a macrocyclic polyamine linked to an acridine moiety capable of catalyzing nucleotide polyphosphates hydrolysis.[35]

Design of the Receptor Molecule 2. Among the various macro(poly)cyclic polyamines that, when protonated, interact electrostatically with nucleotide polyphosphates,[15a,16b,d,21-23] the hexaazamacrocycle [24]-N_6O_2 **1** has been shown to strongly bind

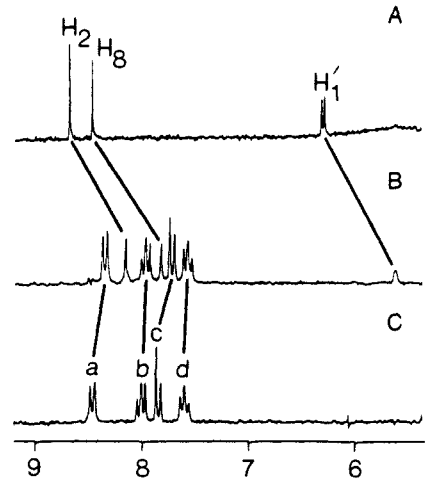

Figure 2. ^1H NMR spectra (aromatic region) of free ATP (A), free macrocycle **2** (C), and the complex (**2**-nH$^+$, ATP^{4-}) (B) at pH 4, 25 °C in D_2O (for peak assignment see structures of ATP and **2**).

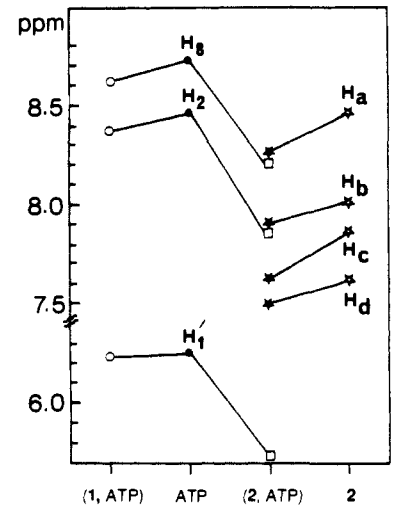

Figure 3. ^1H NMR chemical shifts (aromatic and the anomeric protons) of free ATP (●), (**1**-nH$^+$, ATP^{4-}) (○), (**2**-nH$^+$, ATP^{4-}) (□, ★) and free **2** (☆) at pH 4, 25 °C in D_2O.

ATP, ADP, and AMP, as well as polyphosphates such as pyrophosphate (PP) and triphosphate (TP).[21,29a] Moreover, compound **1** also catalyzes both the hydrolysis[29-31,34] and the synthesis[30,32] of nucleotide polyphosphates. The interesting feature of **1** in the catalysis of P–O–P bond cleavage as well as formation processes was found to be the coexistence, at neutral pH, of ammonium binding sites and of a reactive amine functionality which acts as nucleophile.[29-32,34] Thus, the receptor–catalyst molecule **1** after binding the substrate (ATP, ADP, or acetylphosphate (AcP)), performs nucleophilic catalysis on the bound species. On the other hand, acridine derivatives[36] associate with the nucleic bases of nucleotides and nucleic acids by stacking[37] and by intercalation.[38] In order to generate a receptor molecule that would interact simultaneously with both the polyphosphate chain of nucleotides and their nucleic base moiety, an acridine derivative was attached via a side arm to the macrocycle **1**, yielding compound **2**. This multifunctional receptor molecule **2** thus combined three functional subunits: a macrocyclic polyamine moiety **1** as anion binding site, an acridine side arm for stacking interaction, and a catalytic amino group in the macrocycle for facilitating phosphoryl-transfer reactions.

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Table I. ^{1}H NMR Chemical Shifts (δ in ppm)[a] of **2** in the Absence and Presence of Equimolar Amounts of Substrate in D_2O, 10^{-3} M, 25 °C, at pH 4 and 7

		acridine moiety				aromatic and anomeric protons of nucleotides[e]				
substrate	pH[b]	a[c]	b[d]	c[c]	d[d]	H-2	H-8	H'-1	H-10	H-11
2·8HCl[f]	4	8.47	8.02	7.87	7.62	–	–	–	–	–
	7	8.47	8.03	7.86	7.62	–	–	–	–	–
ATPg	4	8.25	7.88	7.58	7.47	8.21 (8.73)	7.90 (8.46)	5.72 (6.24)	–	–
	7	8.17	7.88	7.58	7.47	8.21 (8.60)	7.84 (8.31)	5.73 (6.19)	–	–
ϵATP	4	8.16	7.83	7.49	7.43	8.91 (9.47)	8.44 (8.92)	5.96 (6.43)	7.89 (8.33)	7.47 (7.96)
	7	8.05	7.79	7.43	7.35	8.75 (9.24)	8.34 (8.69)	5.96 (6.34)	7.72 (8.08)	7.29 (7.66)
ADP	4	8.27	7.98	7.75	7.57	8.13 (8.68)	8.77 (8.43)	5.75 (6.23)	–	–
AMP	4	8.26	7.98	7.81	7.55	8.35 (8.70)	8.08 (8.39)	6.02 (6.22)	–	–
TP[h]	4	8.36	7.97	7.74	7.60	–	–	–	–	–
AP_2A	4	8.19	7.93	7.69	7.51	8.05 (8.34)	7.87 (8.22)	5.76 (6.06)	–	–
AP_4A	4	8.16	7.79	7.50	7.40	7.97 (8.83)	7.80 (8.50)	5.58 (6.04)	–	–

[a] δ with respect to Bu^{t}OH as internal standard. [b] pH of the solution adjusted to desired values with 5 M NaOH or HCl solutions. [c] Doublet. [d] Triplet. [e] Values in parentheses are those observed for free substrates. [f] δ values for the CH_2 groups: at pH 4, 2.15 ($CH_2CH_2CH_2$); 2.71–3.38 (CH_2N); 3.84 (CH_2O); 4.25 (CH_2N-acridine); at pH 7, 2.15; 2.69–3.21; 3.73; 4.26. [g] In the presence of **1** at pH 4, δ 8.59, 8.38 and 6.22 for H-2, H-8, and H'-1 of ATP, respectively. [h] δ values for the CH_2 groups: 2.28 ($CH_2CH_2CH_2$); 3.00–3.96 (CH_2N); 3.88 (CH_2O); 4.22 (CH_2N–acridine).

Table II. Competition Experiments. ^{1}H NMR Chemical Shifts[a] (δ in ppm) of **2** in the Absence and Presence of Equimolar Amounts of ATP, ϵATP, and/or **1** in D_2O, 10^{-3} M, 25 °C, at pH 7.0

substrates	acridine moiety				aromatic and anomeric protons of nucleotides				
and competitors	a[c]	b[d]	c[c]	d[d]	H-2	H-8	H'-1	H-10	H-11
2	8.47	8.03	7.86	7.62	–	–	–	–	–
(**2**, ATP)	8.17	7.88	7.58	7.47	8.21 (8.60)	7.84 (8.31)[d]	5.73 (6.19)[d]	–	–
(**2**, ϵATP)	8.07[f]	7.84	7.55[f]	7.45[f]	8.95[g]	8.52[h]	6.13[i]	786	7.45
(**2**, **1**, ATP)	8.05	7.79	7.43	7.35	8.75 (9.24)[e]	8.39 (8.69)[e]	5.96 (6.34)[e]	7.72 (8.08)[e]	7.29 (7.66)[e]
(**2**, ϵATP, ATP)	8.30	7.93	79.68	7.54	8.24	7.87	5.75	–	–
(**2**, **1**, ϵATP)	8.16	7.83	7.47	7.43	8.91	8.44	5.96	7.89	7.47

[a] δ with respect to Bu^{t}OH as internal reference. [b] Doublet. [c] Triplet. [d] Values in parenthees for free ATP. [e] Values in parentheses for free ϵATP. [f] Poorly defined due to overlapping with other signals and thus δ values are less accurate. [g] For ATP δ = 8.45 ppm. [h] For ATP δ = 8.10 ppm. [i] For ATP δ = 6.01 ppm.

Synthesis of Compound 2. The strategy for the synthesis of **2** consisted in the preparation of the parent pentatosylhexaaza macrocycle **4**[39] to which side arm bearing a terminal amino group may be attached. The intercalator group, an acridine derivative, was then introduced and finally the amine functions were deprotected. The synthetic route developed is flexible and general and permits variation in the side-arm length controlling the distance between the macrocyclic moiety and the intercalant derivative, as well as the introduction of different intercalating agents.

The synthesis of **4** in 10 steps has been reported previously.[39] Treatment of **4** with acrylonitrile in tetrahydrofuran (THF) gave **5** (94% yield) which was reduced to the amino compound **6** by B_2H_6/THF (61% yield). The condensation of **6** with 9-chloroacridine[40] in phenol afforded compound **7** (44% yield) which was deprotected by treatment with HBr/AcOH in the presence of phenol, yielding the hydrobromide salt of **2**. The hydrochloride salt of **2** was prepared in 90% yield by addition of HCl to the free base **2**.

Results

^{31}P NMR Studies. In the presence of **2** at pH 7 in D_2O/H_2O (1/9) solution, the ^{31}P NMR signals of ADP were upfield shifted by 1.16 ppm for P_β and 1.83 ppm for P_α. Addition of 1 equiv of **2** to a 10^{-2} M solution of ATP at pH 7 caused a slight downfield shift of the P_α signal (–0.07 ppm) whereas both the central P_β and the terminal P_γ signals of ATP were significantly shifted upfield by 1.32 and 2.22 ppm, respectively (Figure 1).

^{1}H NMR Studies (Tables I and II). Addition of 1 equiv of ATP to a 10^{-3} M solution of **2** at both pH 4 and pH 7 in D_2O and at room temperature caused significant upfield shifts of the aromatic protons of the acridine moiety of **2**[41] as well as of the H-2, H-8, and the anomeric proton H'-1 of ATP. At pH 4, upfield shifts of 0.22, 0.14, 0.29, and 0.15 ppm were observed for H_a, H_b, H_c, and H_d, respectively; similarly the H-2, H-8, and H'-1 signals of ATP were also shifted upfield by 0.52, 0.56, and 0.52 ppm,

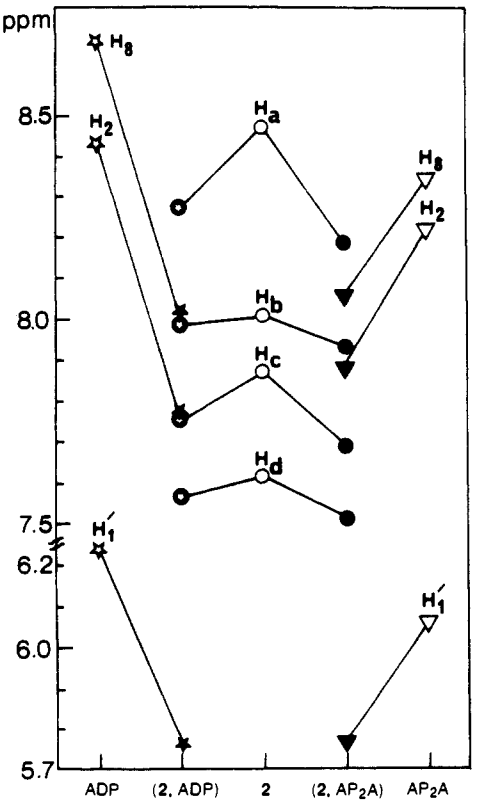

Figure 4. ^{1}H NMR chemical shifts (aromatic and anomeric protons) of ADP^{3-} (☆), (**2**-nH$^+$, ADP^{3-}) (★, ●), **2**-nH$^+$ (○), (**2**-nH$^+$, AP_2A^{2-}) (●, ▼) and AP_2A^{2-} (▽) at pH 4, 25 °C in D_2O.

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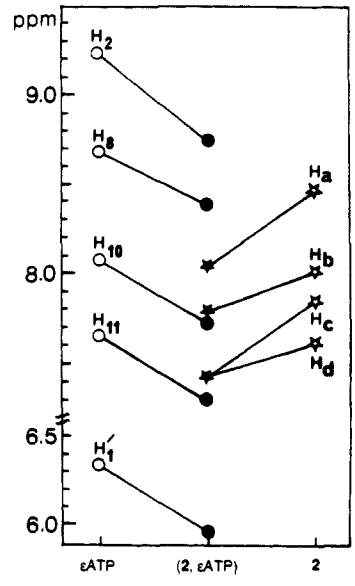
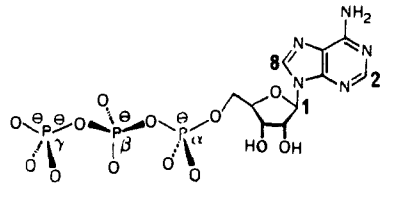


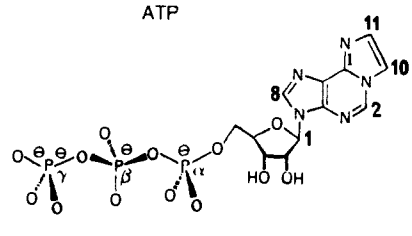
Figure 5. ^{1}H NMR chemical shifts (aromatic and anomeric protons) of εATP (○), (**2**-*n*H$^+$, εATP^{4-}) (●, ★) and **2**-*n*H$^+$ (☆) at pH 4, 25 °C, in D_2O (for peak assignment see structures of εATP and **2**).

respectively (Table I; Figures 2 and 3). On the other hand, the CH_2 protons signals of the macrocyclic part of **2** where downfield from 2.15 ($CH_2CH_2CH_2$), 2.71–3.38 (CH_2N), 3.84 (CH_2O), and 4.25 ppm (CH_2N-acridine) in the free compound **2** to 2.48, 3.11–3.71, 3.94, and 4.34 ppm, respectively, in the presence of ATP. Similarly, addition of 1 equiv of AMP, ADP (Figure 4), AP_2A (P^1,P^2-bis(5′-adenosyl)pyrophosphate) (Figure 4), or AP_4A (P^1,P^4-bis(5′-adenosyl) tetraphosphate) dinucleotides to a solution of **2** at pH 4 induced substantial upfield shifts of the aromatic protons of **2** as well as H-2, H-8, and H′-1 protons of the mono- or dinucleotides (Table I).

Binding of εATP (1,N^6-ethenoadenosine 5′-triphosphate)[42] by **2** at pH 4 and 7 was studied by ^{1}H NMR spectroscopy. At pH 4 in the presence of 1 equiv of εATP, upfield shifts of 0.19–0.38 ppm were observed for H_a, H_b, H_c, and H_d protons of **2**. The H-2, H-8, H′-1, H-10, and H-11 protons of εATP were also upfield shifted by ca. 0.5 ppm (Table I). At pH 7, similar upfield shifts of the aromatic protons of the acridine part of **2** and of the ethenoadenine moiety and the anomeric proton H′-1 of εATP were measured (Table I, Figure 5).



ATP



εATP

Addition of triphosphate (TP) to a 10^{-3} M solution of **2** at pH 4 caused significant downfield shifts of the CH_2 groups of the macrocyclic moiety of **2**, whereas signals corresponding to the acridine part were shifted slightly upfield (Table I).

Competition Experiments. The results of competition experiments between **1** and **2** for either ATP or εATP, and between ATP and εATP for **2** at pH 7, observed by ^{1}H NMR spectroscopy, are summarized in Table II. Addition of 1 equiv of **1** to a 10^{-3} M solution of **2** and ATP (where all ATP is bound) caused downfield shifts of 0.05–0.13 ppm for the H_a, H_b, H_c, and H_d protons and of ca. 0.03 ppm for the H-2, H-8, and H′-1 protons of ATP; simultaneously, the signals of the aromatic protons of **2** and those of H-2, H-8, H′-1, H-10, and H-11 protons of εATP were shifted slightly downfield by 0.00–0.11 and 0.00–0.18 ppm, respectively. Finally, addition of 1 equiv of ATP to a 10^{-3} M solution of **2** and εATP induced downfield shifts of the aromatic protons of **2** by 0.02–0.12 ppm and of the proton signals of εATP by 0.13–0.20 ppm, whereas the H-2, H-8, and H′-1 protons of ATP were upfield shifted by 0.15–0.21 ppm, when compared to the free ATP signals (Table II).

Variable-Temperature Study. The ^{1}H NMR spectra of **2**, ATP and (**2**, ATP) complexes were recorded at 5 and 25 °C in D_2O at pH 4. The aromatic proton signals of **2** were shifted slightly downfield by 0.01–0.03 ppm when the temperature was decreased from 25 to 5 °C. The H-2, H-8, and H′-1 signals of ATP also shifted downfield by 0.01–0.02. On the other hand, for the (**2**-*n*H$^+$, ATP^{4-}) complexes, the chemical shift changes observed when the temperature was lowered from 25 to 5 °C, were ca. 0.02 ppm for the H_a, H_b, H_c, and H_d signals of **2**; the H-2 proton of ATP was shifted slightly downfield by 0.02 ppm, whereas the H-8 and H′-1 protons were upfield shifted by ca. 0.04 ppm.

Fluorescence Study (Tables III and IV). Absorption Properties of 2. Whereas the parent compound **1** does not absorb light in the near ultraviolet or visible region, compound **2** possesses a strong acridine chromophore[43] (λ_{max} = 378 nm, ϵ_{max} = 8.5 × 10^3; 408 nm, 1.0 × 10^4; 429 nm, 6.4 × 10^3). The neutral aqueous solution of **2** is yellow. Upon addition of ATP, the main bands at 408 nm and at 429 nm undergo bathochronic shifts of 2 nm to 410 nm and 431 nm with a slight hypochromic effect (for λ_{max} = 410 nm, ϵ_{max} = 9.5 × 10^3). Neither ADP, AMP, nor triphosphate (TP), diphosphate (PP), or phosphate (P) affect the absorption spectra of **2**.

Emission Properties of 2 (Table III, Figure 6). In dilute aqueous solution, **2** and the model compound **8**, like 9-aminoacridine **9**, emit a strong blue fluorescence.[44] Excitation of **2** at 408 nm produces a fluorescence emission at 450 nm. No changes in the intensity or the wavelength of the emission were observed upon changing the pH of the solution from 4.0 to 7.6. However, at higher pH values, due to the deprotonation of the acridine moiety of **2**, a significant increase of both the wavelengths (465 nm, 485 nm) and the relative fluorescence intensities was observed, a behavior already reported for 9-aminoacridine **9**.[44]

Binding of AMP, ADP, and ATP. In the presence of ATP and ADP, a bathochromic shift of 2 nm was observed for the emission wavelength (452 nm) of **2**, whereas with the other substrates studied, no change was measured. In contrast, all these substrates led to significant changes in the emission intensity (Table III, Figure 6. Addition of equimolar amounts of P, PP, and TP had almost no effect on the relative fluorescence intensity of **2**, **8**, and **9** at both pH 4.0 and 7.6 (Figure 6).

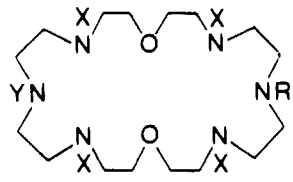
Although addition of 1 equiv of ATP and ADP to a 10 μM solution of **2** caused significant enhancement of the emission intensity at pH 4.0 and 7.6, their presence had no effect on the emission of **8** and **9** (Figure 6). The presence of 1 equiv of ATP and CTP caused an enhancement of the fluorescence of **2**, whereas GTP induced a quenching of its emission (Figure 6).

Binding of εATP (Tables III, IV). Since ATP fluorescence is weak, ethenoadenosine triphosphate (εATP),[42] which emits strongly at 410 nm when exicted at 300 nm, was studied as analogue of ATP. In the presence of 1 equiv of **2**, the emission of εATP was considerably quenched (40%) whereas the emission

(42) (a) Barrio, J. R.; Secrist, J. A., III; Leonard, N. J. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 597–604. (b) Secrist, J. A., III; Barrio, J. R.; Leonard, N. J. *Science* **1972**, *175*, 646–647. (c) Secrist, J. A., III; Barrio, J. R.; Leonard, N. J.; Weber, G. *Biochemistry* **1972**, *11*, 3499–3506.

(43) Nakamizo, N. *Spectrochim. Acta* **1966**, *22*, 2039–2053.

(44) Mataga, N.; Kubota, T. *Molecular Interactions and Electronic Spectra*; Marcel Dekker Inc.: New York, 1970.



(1) X=Y=R=H

(2) X=Y=H, R=$(CH_2)_3NHZ$

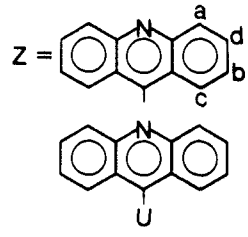
(3) X=H, Y=PO_3^{2-}, R=$(CH_2)_3NHZ$

(4) X=Y=Ts, R=H

(5) X=Y=Ts, R=$(CH_2)_2CN$

(6) X=Y=Ts, R=$(CH_2)_3NH_2$

(7) X=Y=Ts, R=$(CH_2)_3NHZ$



(8) U=NH_2

(9) U=$NH(CH_2)_3N(CH_3)_2$

of **2**, when excited at 408 nm was almost unchanged.

Binding of Dinucleotides (Tables III). The effect of AP_2A and AP_4A on the emission of **2** was investigated at pH 4.0. Although an equimolar amount of AP_2A produced no change, a 100-fold excess enhanced significantly the emission of both **2** and **8**. Addition of 1 equiv of AP_4A had no effect on the emission of **8**, but it enhanced the emission of **2**.

Competition Experiments. The results of competition experiments between ϵATP and either ATP or TP substrates for ligand **2** or between ligands **1** and **2** for either ϵATP or ATP as well as between ATP and TP for **2** at pH 7.6 are given in Table IV. Starting with a solution of **2** and ϵATP (10 μM each) addition of 1–10 equiv of **1** caused no change in the emission of ϵATP or of **2**; when 1 equiv of ATP was added, the emission intensities of both ϵATP and **2** were slightly enhanced, and the increase was significantly larger with a 10-fold excess of ATP; addition of 1–10 equiv of TP did not change the emission of **2** but enhanced that of ϵATP. Addition of 1 equiv or of a 10-fold excess of **1** or TP to an equimolar mixture of **2** and ATP decreased the emission of **2**.

Stoichiometry of ATP Binding. The emission intensity I_f of **2** at 452 nm was measured at pH 4, in the presence of increasing amounts of ATP. The plot of I_f of **2** vs the ratio R of ATP to ligand **2** (Figure 7) indicates the formation of a 1/1 complex of **2**/ATP.

Variable-Temperature Study. The emission of a 10 μM solution of **2** in the presence of 1 equiv ATP at pH 4 was measured at 1 and 23 °C. The emission intensity of the free ligand **2** at 450 nm increased by a factor of 2 on cooling to 1 °C. Similarly, the emission of **2** in the presence of ATP at 452 nm also doubled when the temperature was increased from 23 to 1 °C.

Catalysis of ADP and ATP Hydrolysis by 2. Hydrolysis of both ATP and ADP in the presence of **2** at pH 7 and 80 °C in D_2O/H_2O (1/9) was followed by ^{31}P NMR spectroscopy. The reaction products of catalyzed ADP hydrolysis were AMP and P. The hydrolysis of ATP in the presence of **2** produced ADP and P at the beginning; after some ADP was generated, it was in its turn hydrolyzed to AMP and P. In both cases, the reaction proceeded, at least in part, through a covalent phosphoramidate intermediate (compound **3**)[30] giving a ^{31}P NMR signal at 10.10 ppm. This intermediate **3** first accumulated and then disappeared. The plot of ATP and ADP disappearance as a function of time gave apparent first-order rate constants of k_{obs} = 0.0015 min^{-1} for ADP and k_{obs} = 0.0126 min^{-1} for ATP (Figure 8).

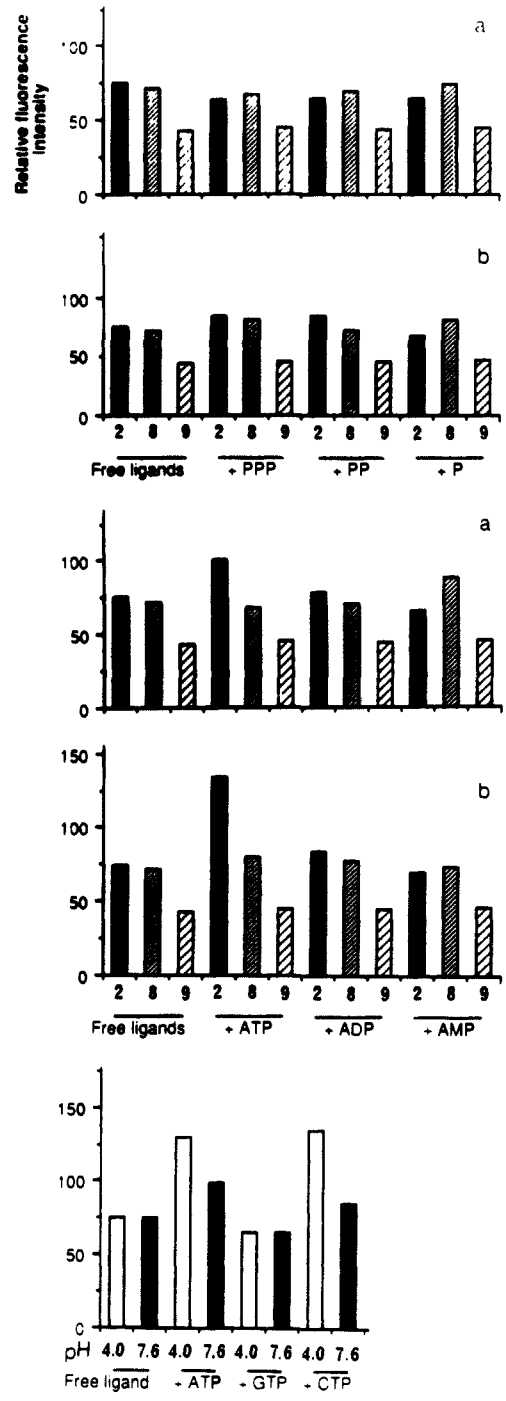


Figure 6. Relative fluorescence intensities of **2**, **8**, and **9** in the absence and in the presence of equimolar concentrations of P, PP, and PPP (top two histograms), or AMP, ADP, and ATP (middle two): (a) pH 7.6, (b) pH 4.0. The bottom histogram shows the emission intensity of **2** in the absence and in the presence of ATP, GTP, and CTP (see Table III).

Interaction with DNA. The electrophoretic migration ability of the supercoiled circular double stranded DNA plasmid pBR322 at pH 7.6 (Tris buffer) on agarose gel was strongly retarded in the presence of 10^{-6} M compound **2**. No retardation of migration was observed in the presence of 9-aminoacridine **8** or compound **1** or an equimolar mixture of both. Compound **2** was not active in the cleavage of pBR322 plasmid.

Discussion

Binding of nucleotide polyphosphates by the protonated macrocycle **2** is investigated by three spectroscopic methods. ^{31}P NMR spectroscopy is used to ascertain interactions between the protonated macrocyclic part of **2** and the negatively charged polyphosphate chains of nucleotides. ^{1}H NMR allows observation

Table III. Relative Fluorescence Emission Intensities (I_{rel}) (±10%) of Acridine Derivatives **2**, **8**, and **9** in the Presence and Absence of Equimolar Amounts of Phosphate Containing Substrates at pH 4.0[a] and 7.6,[b] H_2O, 10 μM, 25 °C

substrates ligands	pH	none[d]	P[d]	PP[d]	TP[d]	AMP[d]	ADP[e]	ATP[e]	εATP[f]	GTP[d]	CTP[e]	AP^2A[d]	AP^4A[d]
2[c]	4.0	75	68	82	84	70 (88)[g]	84	135	–	65	140	76 (124)[g]	240
	7.6	75	65	65	65	68 (80)[g]	78	100	73	65	85	–	–
8[c]	4.0	72	80	72	80	73	76	80		76	–	75 (124)[g]	75
	7.6	71	75	71	66	87	70	66	–	–	–	–	
9[h]	4.0	42	44	45	46	45	45	45	–	44	–	–	
	7.6	42	44	45	45	44	45	–	–	–	–	–	–

Table IV. Competition Experiments. Relative Fluorescence Intensities (I_{rel}) (±10%) of **2** and εATP in the Presence and Absence of **1**, ATP, and TP at pH 7.6,[a] in H_2O, 25 °C

$\lambda_{excitation}$ (nm)	free species[b]		starting complex[b]	additive to complex[c]			
	2	εATP			**1**	ATP	TP
300	16[d]	516[e]	(**2**, εATP)[d]	230	230[e] (230)	240[e] (288)	262[e] (296)
408	75[d]	–	(**2**, εATP)	73	73 (73)	83 (110)	75 (75)
408	–	–	(**2**, ATP)[f]	100[f]	92[f] (86)	–	88[f] (80)

[a] TrisOAc (0.1 M). [b] [εATP] = [ATP] = [**2**·8HCl] = 10 μM. [c] [**1**·6HCl] = [ATP] = [TP] = 10 μM; values in parentheses for [**1**·6HCl] = [ATP] = [TP] = 100 μM. [d] Acridine emission at 450 nm. [e] εATP emission at 410 nm. [f] Acridine emission at 452 nm.

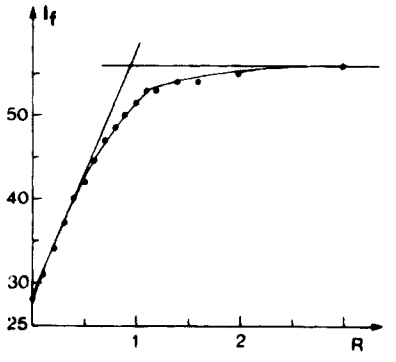


Figure 7. Relative emission intensity (I_f) of **2** (excitation at 408 nm, emission at 452 nm) as a function of increasing ratio R = ATP/**2** in H_2O at pH 4, 25 °C; [**2**] = 10^{-5} M.

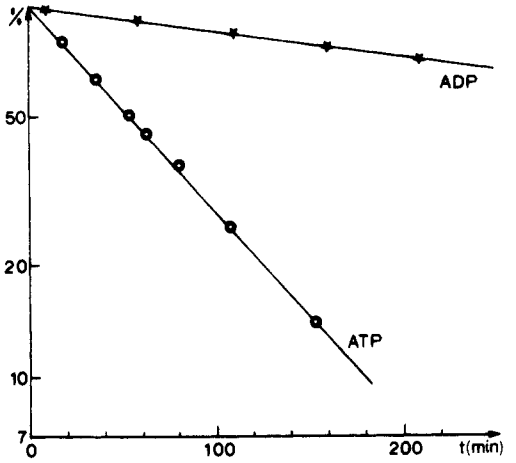


Figure 8. Semilog plot of the percentage of remaining ATP and ADP in independent runs vs time (min) at 80 °C and pH 7.0 in the presence of equimolar concentrations of macrocycle **2**.

of interactions between the macrocyclic moiety of **2** and the polyphosphate chain as well as between the acridine part of **2** and the nucleoside moiety of the nucleotide. Finally, fluorescence emission spectrophotometry serves to demonstrate stacking interactions between the acridine part of **2** and the nucleic base. Since ATP shows weak fluorescence, εATP,[42] which emits strongly, is used as its analogue. In this case, in addition to ^1H NMR studies, changes in the fluorescence intensity of both the ligand **2** and εATP are measured. Compounds **8** and **9** are studied as reference compounds to mimic the pendant side arm of **2** bearing the 9-aminoacridine derivative, whereas the parent compound **1** serves an analogue of its macrocyclic moiety.

Binding of the Polyphosphate Chain. The formation of complexes of the type (**2**-nH$^+$, AXP^{m-}) with X = D or T and m = 3 or 4 leads to upfield shifts of the ^{31}P NMR signals of nucleotides. Ligand **2** induces almost the same shifts as the parent compound **1**[21,29a] (Figure 1). Furthermore, similar downfield shifts of the CH_2 groups protons signals of the macrocyclic moiety of **2** are obtained in the presence of either ATP or TP (Table I). These observations demonstrate the binding of the polyphosphate chain by protonated **2**.

Binding of the Nucleic Base. Stacking interactions between the acridine part of **2** and the nucleic base of nucleotide are observed by ^1H NMR and by fluorescence emission spectroscopies. Indeed, the binding of the nucleotides AMP, ADP, or ATP by the protonated compound **2** causes significant upfield shifts of the H-2, H-8, and H′-1 signals (Table I, Figures 2, 3). On the other hand, the aromatic protons of the acridine moiety of **2** are also shifted upfield, although to a lesser extent, in the presence of nucleotides (Table I, Figures 2–4). Similarly, the binding of εATP by **2**-nH$^+$, induces upfield shifts of the aromatic protons of **2** as well as those of the 1,N^6-ethenoadenine and of the anomeric protons of εATP (Table I, Figure 5). The same type of behavior is observed for the symmetrical AP_2A (Figure 4) and AP_4A dinucleotides.

The strong acridine fluorescence[44] of compound **2** remains constant when the pH of the solution is increased from 4.0 to 7.6, probably indicating that the protonation state of the acridine moiety of **2** is unchanged (Table III, Figure 6). This is confirmed by ^1H NMR which shows no difference between the chemical shifts of the aromatic protons of **2** at pH 4.0 or 7.0 (Table I).

In the presence of 1 equiv of (poly)phosphate containing substrates, the emission intensities of both model compounds **8** and **9**, at 10^{-5} M concentration, are almost unaffected; in the presence of 100-fold excess of AP_2A, the emission of **8** is enhanced by a factor of 1.7 (Table III). These observations indicate that at a 10^{-5} M concentration, interactions between the intercalating agents **8** and **9** and the nucleotides are rather weak so that no stacked complexes are detected. In contrast, the significant enhancement of the emission of **2** at both pH 4.0 and 7.6, in the presence of mono- or dinucleotides (except in the case of GTP which quenches slightly the emission), indicates stacking of the acridine part of **2** with the nucleic bases. It was shown previously that adenine and cytidine nucleotides enhance the emission of acridine derivatives whereas guanine nucleotides quench it by stacking interactions.[45] Since the emission of **2** is not altered by the presence of P, PP or TP (Table III, Figure 6), the binding of these anionic

(45) Georghiou, S. *Photochem. Photobiol.* **1975**, *22*, 103–109. (b) *Ibid.* **1976**, *24*, 417–423.

substrates to the macrocyclic moiety of **2** does not affect the emission properties of its acridine group. Furthermore, when the model compound **9**, the parent macrocycle **1**, and ATP are all present simultaneously in solution at 10^{-5} concentrations at pH 4.0 or 7.6, no change in the emission of **9** is observed. This shows that no interaction with **9** takes place, although due to the high affinity of **1** for ATP, (**1**-nH$^+$, ATP^{4-}) complexes are present in solution.[21,29a] It demonstrates the importance of the covalent linkage between the anion binding site **1** and the acridine derivative within the receptor molecule **2**, leading to simultaneous interactions with the polyphosphate chain and the nucleic base of nucleotides.

The marked increase in emission intensity observed on addition to ATP to **2** (especially around pH 4, Figures 6 and 7), together with the high stability of the complex formed, make compound **2** a *sensitive fluorescent probe* for ATP (as well as CTP) detection, displaying also *selectivity* for ATP vs GTP (which slightly decreases the emission of **2**, Figure 6, bottom).

Stability of ATP Complexes with 2. The protonated parent compound **1** binds strongly and selectively ADP and ATP in aqueous solution. The stoichiometries of these complexes are 1/1, and their stability constants were determined by pH-metric titration.[21,29a] For binding of ATP by **1**-nH$^+$, the stability constants [(log K_S) 11.00 for (**1**-6H$^+$, ATP^{4-}), 8.15 for (**1**-5H$^+$, ATP^{4-}), and 4.80 for (**1**-4H$^+$, ATP^{4-})] were calculated.[21] At pH 7, the dominant species in solution were found to be the tetra and the pentaprotonated complexes. The competition experiments (Table II) allow to check whether **2**, which bears an additional binding site for adenine, forms stronger complexes with ATP than **1** at pH 7. Observation by ^{1}H NMR of the competition between **1** and **2** for ATP indicates clearly that **2** forms stronger complexes with ATP than **1** by at least a factor of 2: indeed, addition of 1 equiv of **1** to the solution containing **2** and ATP causes only small downfield shifts of the aromatic protons of **2** and of the H-2, H-8, and H′-1 protons of ATP. Similarly, the slight downfield shifts of the acridine protons of **2** and of ethenoadenine, and of the anomeric protons of ϵATP, observed on addition of an equimolar amount of **1** to a solution of **2** and ϵATP, indicate the formation of stronger complexes between **2** and ϵATP by a factor of about 2 than between **1** and ϵATP. Furthermore, competition between ATP and ϵATP for **2** shows clearly that the latter is bound by a factor of ca. 5 more strongly, probably due to greater stacking interactions of its acridine moiety with the larger ethenoadenine than with adenine.

Fluorescence emission studies confirm these results (Table IV), since on addition of 1 equiv of **1**, the emission of (**2**-nH$^+$, ATP^{4-}) is only slightly quenched. Addition of even a 10-fold excess of **1** does not restore the emission intensity of the free ligand **2**. On the other hand, competition between ATP and TP for **2** indicates that TP, although being more highly charged than ATP, forms weaker complexes with **2** by at least a factor of 2. Similarly, ϵATP binds more strongly to **2** than to **1**, since addition of 1–10 equiv of **1** has no effect on the emission of either **2** or ϵATP. Compound **2** also binds ϵATP stronger than ATP, because addition of ATP to a solution of **2** and ϵATP causes only a slight enhancement of the emission of ϵATP and **2**.

In the presence of TP, again an enhancement of the emission of ϵATP is observed, although even a 10-fold excess of TP does not restore the full emission of free ϵATP, indicating that **2** binds ϵATP more strongly than TP. At the same time the emission of **2** remains unchanged, as expected since TP itself does not alter the emission of **2** (see above).

Geometry of the Complexes Formed by 2 with Nucleotide Phosphates. The stoichiometry of ATP binding by **2** is found to be 1/1 (Figure 7). Assuming that the (poly)phosphate chain of nucleotides interacts with the positively charged ammonium centers of the macrocyclic moiety of **2**, two types of complexes may be envisaged: complexes in which the relative orientation of the two partners is such as to allow interaction between the acridine part of **2** and the nucleic base of nucleotides (syn complexes) or complexes in which the stacking cannot take place (anti complexes) (Figure 9). If a mixture of such complexes is present in solution, changing the temperature should modify their proportion. Variable-temperature ^{1}H NMR studies show that proton signals of the free compound **2**, free ATP, or (**2**-nH$^+$, ATP^{4-}) complexes are only slightly affected by increasing the temperature from 5 to 25 °C; shifts of less than 0.03 ppm are observed for the aromatic protons of **2** and H-2, H-8, and H′-1 protons of ATP. Similarly, the ratios of the emission intensity of (**2**-nH$^+$, ATP^{4-}) complexes to that of the protonated free ligand **2**-nH$^+$ are nearly identical at 23 °C (2.29 ± 0.2) and at 1 °C (2.11 ± 0.2). These results indicate that the proportion of different complexes which may exist in solution is not significantly affected by lowering the temperature by ca. 20 °C.

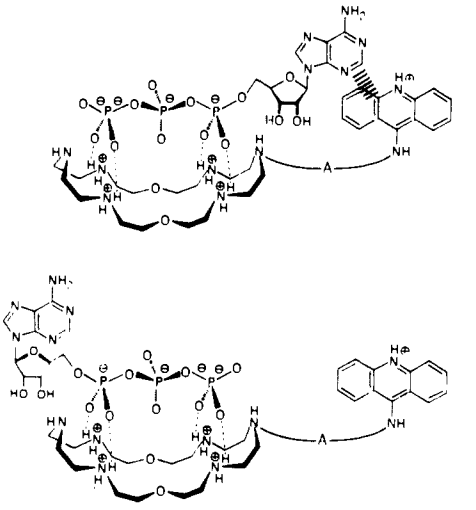


Figure 9. Schematic representation of the possible syn (top) and anti (bottom) complexes formed by **2**-5H$^+$ with ATP^{4-} [A = $(CH_2)_3$]. Electrostatic and stacking interactions are represented by --- and ||||, respectively.

Furthermore, it is observed that the aromatic protons of **2** are similarly shifted in the presence of ADP, ATP, or AP_2A, whereas the shifts observed for H-2, H-8, and H′-1 protons of the symmetrical substrate AP_2A are about the half of those obtained for ADP or ATP (Table I, Figure 4). On the basis of these observations and the facts that **2**-nH$^+$ forms stronger complexes with ATP than does **1**-nH$^+$ and that it binds better ATP than TP, it seems reasonable to propose that the dominant species in solution are complexes of syn type in which the polyphosphate chain and the nucleic bases of nucleotides interact respectively and simultaneously with the macrocyclic moiety and the acridine derivative of **2**-nH$^+$ (Figure 9).

Catalysis of ATP and ADP Hydrolysis. Compound **2** catalyzes the hydrolysis of both ATP and ADP, the hydrolytic reactions being first order with respect to the complexes formed (Figure 8) and accelerated by factors of ca. 3 for ADP and 9 for ATP. The mechanism of nucleotide hydrolysis catalyzed by **1** has been previously investigated in detail.[29-34] Since **2** behaves similarly to **1**, it seems reasonable to assume the same reaction mechanism for **2**. Indeed, after binding ADP or ATP, protonated compound **2**, like compound **1**, undergoes a phosphorylation process in which the terminal phosphoryl group of the substrate is transferred to the unprotonated centrally located secondary amine of the macrocyclic moiety of **2**, yielding the phosphoramidate **3**. The latter, as observed by ^{31}P NMR, first accumulates and then is hydrolyzed, leading to the starting macrocycle **2** which participates in another catalytic cycle (Figure 10). Since the ^{31}P NMR chemical shift observed for the intermediate **3** (10.10 ppm) is almost identical to that observed for the intermediate formed by **1** (10.00 ppm), the location of these phosphoramidates should be the same. As for compound **1**, in the case of ATP hydrolysis, no cleavage of the P–O–P bond yielding AMP and PP is observed. Compound **2** is less effective than **1** in catalyzing either ATP or ADP hydrolysis. This is in agreement with our previous finding that when an amino-ethyl side arm is attached to the central amino group of one of the diethylenetriamine subunits of **1**, the catalytic ability of the latter is significantly reduced for both the ADP and ATP hydrolysis.[34] Nevertheless, **2** accelerates the hydrolysis of ADP

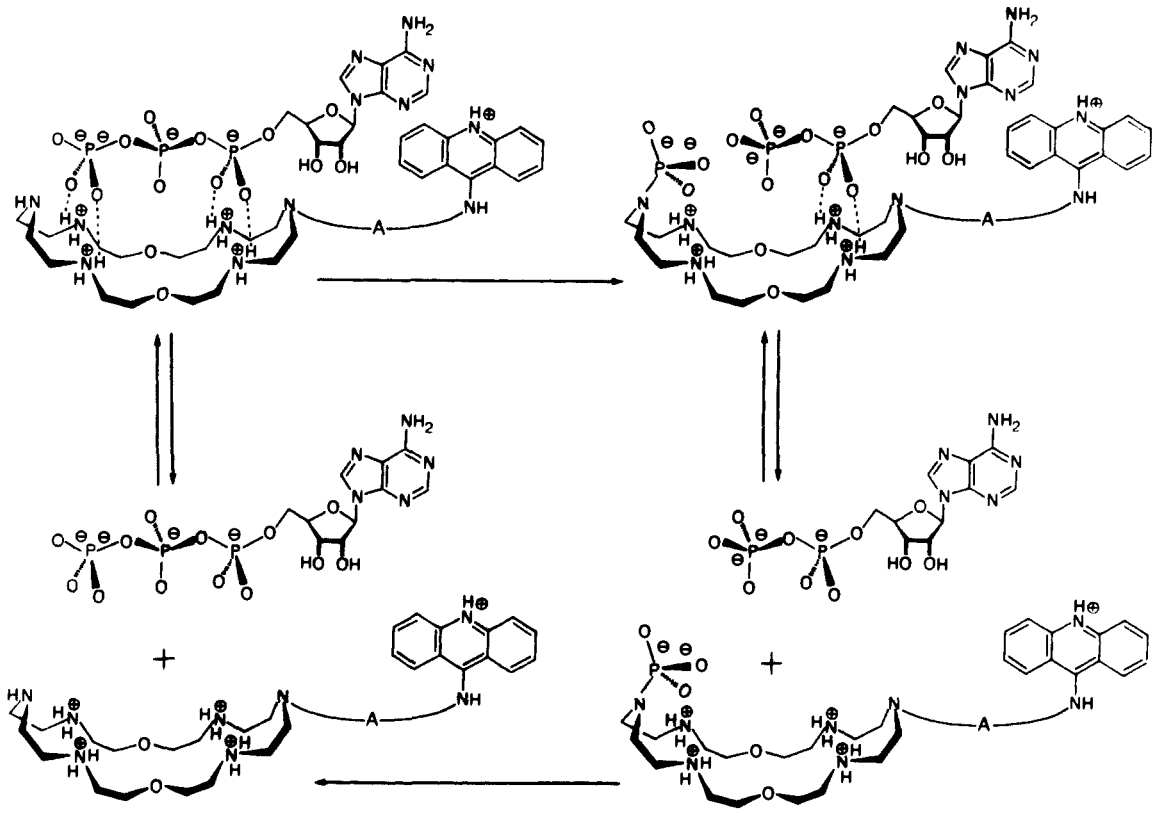


Figure 10. Schematic representation of the catalytic cycle for ATP hydrolysis by the macrocyclic compound **2** following the nucleophilic pathway.

substantially less than that of ATP, demonstrating a greater *catalytic selectivity* between ATP and ADP than what was observed for **1**.[29a] Indeed, for compound **1** at pH 7 and 80 °C, the ratio of the observed first-order rate constants for ATP and ADP hydrolysis is only 2.7.[29] Under the same conditions, a factor of 8.4 is obtained for **2**. However, the derivative of **1** bearing a hydroxyethyl chain (**1** with X = Y = H, R = CH_2CH_2OH) displays a selectivity of about 6.9 for the same reactions.[34] Thus, although the acridine site clearly participates in the interaction between ATP and **2**, this additional binding has little effect on the ATP/ADP selectivity. It may however be only an apparent lack of effect; indeed, whereas **2** binds ATP and ADP preferntially in one orientation, the hydroxyethyl compound may be expected to form comparable amounts of both complexes (Figure 9).

Binding to DNA. Analysis of band displacement in gel electrophoresis indicates that compound **2** binds strongly to the supercoiled circular double stranded DNA plasmid pBR322 at pH 7.6 and 10^{-6} M concentration probably via double interaction involving both intercalation of the acridine moiety of **2** within the base pairs and electrostatic attraction between the macrocyclic polyammonium anion receptor part of **2** and the phosphate groups of DNA. This is reasonable since mono- and polyintercalators built on biogenic polyamines (spermine, spermidine) have been shown to bind to DNA.[46] Compound **2**, although interacting strongly with DNA, is not able to induce its chemical or photochemical cleavage.

Conclusion

Protonated compound **2** displays *double recognition* in binding nucleotides by simultaneous attractive electrostatic interactions between the polyphosphate chain of the substrate and the anion receptor macrocyclic polyammonium moiety of **2** as well as between the acridine intercalant group of the latter and the nucleic base (Figure 9). Due to this simultaneous binding, protonated **2** forms stronger complexes with nucleotides than the parent macrocycle **1** which does not contain the acridine side arm. Furthermore, as a consequence of more pronounced stacking interactions, **2** binds ϵATP stronger than ATP. The emission enhancement of **2** induced by the binding of ATP also makes this compound a sensitive and selective fluorescent probe for this nucleotide triphosphate.

Compound **2**, like **1**, accelerates the hydrolysis of ATP and ADP via nucleophilic catalysis, the hydrolytic reactions proceeding, at least in part, through the formation of the covalent phosphoramidate intermediate **3**. Moreover, **2** shows greater hydrolytic selectivity than **1** between ATP and ADP. Compound **2** binds strongly to DNA, but does not cleave it. DNA cleavage might be achieved by replacement of the acridine moiety of **2** by photo and/or electroactive intercalating derivatives. Protonated **2**, although interacting with the nucleic base of nucleotides by stacking, shows no significant recognition between them. In order to perform molecular recognition of the nucleic bases, more specific information features than simple stacking interactions, such as complementary hydrogen bonding, must be introduced. The elaboration of such receptor molecules is under current investigation.

Experimental Section

General. Melting points (mp) are uncorrected. ^1H and ^{13}C NMR spectra are recorded on a Bruker SY 200 spectrometer. Chemical shifts are given in ppm with respect to tetramethylsilane in $CDCl_3$ and *tert*-

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butyl alcohol in D_2O as internal standards. Mass spectra (MS) and elemental analyses were performed by the Service de Spectrométrie de Masse and by the Service de Microanalyse, Institut de Chimie, Strasbourg.

Materials. Compound **1**[52] was used as its hexahydrochloride salt, **9** was prepared as previously described,[53] **8** was commercially available from Aldrich Chemical Co. The commercially available chemicals used were of reagent grade. The Na_2 salts of adenosine triphosphate, of adenosine diphosphate, and of adenosine monophosphate and the Li_3 salt of P^1, P^4-bis(5'-adenosyl) tetraphosphate were purchased from Boehringer, Mannheim. The Na salt of guanosine-triphosphate, cytidine-triphosphate P^1,P^2-bis(5'-adenosyl)pyrophosphate and of 1,N^6-etheno-adenosine 5'-triphosphate were purchased from Sigma.

Synthesis of Macrocyclic Compound 2. 7-(2-Nitriloethyl)-4,10,16,19,22-pentakis(*p*-tolylsulfonyl)-1,13-dioxa-4,7,10,16,19,22-hexaazacyclotetracosane (5). In a 100-ml round-bottom flask, compound **4** (6 g, 5.37 mmol), acrylonitrile (10 mL), and dry THF (50 mL) were refluxed for 11 days. The mixture was evaporated to dryness and dried under vacuum for 14 h, yielding a colored glass. The pure compound **5**, a colorless glass, was isolated as the first moving spot by column chromatography (silica gel, $MeOH/CH_2Cl_2$ 0–0.5%) (5.90 g, 94% yield): ^1H NMR ($CDCl_3$) 2.41 (s, 15 H, CH_3), 2.80 (m, 6 H, CH_2N), 3.13–3.39 (brm, 22 H, CH_2NTs, CH_2CN), 3.57 (brm, 8 H, CH_2O), 7.22–7.35, 7.59–7.76 (m, 20 H, aromatic); ^{13}C NMR ($CDCl_3$) 16.6 (CH_2CN), 21.3 (CH_3), 47.9, 48.8, 49.1, 49.6, 49.7, 50.0, 53.4 (CH_2N), 69.5, 69.9 (CH_2O), 119.1 (CN), 126.9, 127.1, 127.2, 129.6, 129.7, 135.4, 136.3, 143.2, 143.4, 143.5 (aromatic). Anal. Calcd for $C_{54}H_{71}O_{12}N_7S_5 \cdot 0.5\ CH_2Cl_2$ (1212.96): C, 53.97; H, 5.98; N, 8.08. Found: C, 53.98; H, 5.85; N, 8.07.

7-(3-Aminopropyl)-4,10,16,19,22-pentakis(*p*-tolylsulfonyl)-1,13-dioxa-4,7,10,16,19,22-hexaazacyclotetracosane (6). In a 250-mL flask, compound **5** (5.70 g, 4.87 mmol) and dry THF (40 mL) were stirred under argon for 15 min; 100 mL of 1 M B_2H_6/THF was added, and the mixture was refluxed for 16 h. The solution was cooled in an ice bath, and the excess B_2H_6 was destroyed carefully by addition of H_2O/THF (50/50). The solution was evaporated to dryness leaving a white solid which was refluxed in 6 N HCl (200 mL) for 6 h. Evaporation left a white solid which was dried under vacuum overnight before it was taken up in CH_2Cl_2 (200 mL) and washed with 2.5 N NaOH (200 mL); the emulsion formed was broken by addition of saturated NaCl solution (100 mL). The organic layer was separated, and the aqueous phase was further extracted with CH_2Cl_2 (2 × 150 mL). The organic layers were combined and dried over $MgSO_4$, and the solvent was removed, leaving a slightly yellowish glass which was dried under vacuum for 24 h. This compound was used in the next step without further purification (3.5 g, 61% yield): ^1H NMR ($CDCl_3$) 1.53 (s, 2 H, $CH_2CH_2CH_2$), 2.39, 2.41 (2s, 15 H, CH_3), 2.68 (m, 6 H, CH_2N), 3.08–3.40 (brm, 24 H, CH_2NTs, CH_2NH_2, NH_2), 3.54 (brt, 8 H, CH_2O), 7.25–7.32, 7.64–7.72 (m, 20 H, aromatic), ^{13}C NMR ($CDCl_3$) 21.5 (CH_3), 30.4 ($CH_2CH_2CH_2$), 40.6 (CH_2NH_2), 47.6, 48.5, 49.5, 49.7, 53.4 (CH_2NTs), 70.0, 70.1 (CH_2O), 127.1, 127.3, 129.8, 129.9, 136.0, 144.3 (aromatic). Anal. Calcd for $C_{54}H_{75}O_{12}N_7S_5 \cdot 0.5\ CH_2Cl_2$ (1216.99): C, 53.79; H, 6.29; N, 8.06. Found: C, 53.76; H, 6.48; N, 8.17.

7-[3-(9-Acridinylamino)propyl]-4,10,16,19,22-pentakis(*p*-tolylsulfonyl)-1,13-dioxa-4,7,10,16,19,22-hexaazacyclotetracosane (7). In a 25-mL round-bottom flask, compound **6** (1 g, 0.85 mmol), phenol (2 g), and 9-chloroacridine[40] (0.182 g, 0.85 mmol) were heated under argon to 80 °C for 6 h. The reaction mixture was allowed to cool to room temperature, and the residue was taken up in CH_2Cl_2 (100 mL) and washed with 2.5 N NaOH (40 mL). The organic layer was separated, and the aqueous phase was further extracted with CH_2Cl_2 (2 × 25 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated to dryness. The pure compound **7** was obtained by column chromatography (silica gel, $MeOH/CH_2Cl_2$ 0–5%) (0.5 g, 44% yield): ^1H NMR ($CDCl_3$) 2.10 (brm, 2 H, $CH_2CH_2CH_2$), 2.27, 2.34 (2s, 15 H, CH_3), 2.85 (brm, 6 H, CH_2N), 3.18–3.40 (brm, 20 H, CH_2NTs), 3.47, 3.48 (2 brs, 8 H, CH_2O), 4.20 (br, 2 H, CH_2NH), 7.12–7.24, 7.47–7.59 (m, 24 H, aromatic), 8.18 (t, 4 H, aromatic); ^{13}C NMR ($CDCl_3$) 21.30, 21.35 (CH_3), 26.5 ($CH_2CH_2CH_2$), 46.8, 48.4, 48.8, 49.1, 49.3, 49.6, 51.2, 53.3 (CH_2N), 69.6 (CH_2O), 112.0, 119.5, 123.2, 125.4, 126.7, 127.0, 127.2, 129.6, 129.7, 133.9, 135.2, 136.3, 139.8, 143.3, 143.5, 143.6, 156.6 (aromatic); MS *m/z* 1351 (M^+), 1195 (M^+ – 1Ts), 1041 (M^+ – 2Ts). Anal. Calcd for $C_{67}H_{82}O_{12}N_8S_5 \cdot 2.5\ CH_2Cl_2$ (1564.05): C, 53.38; H, 5.60; N, 7.16. Found C, 53.60; H, 5.54; N, 7.53.

7-[3-(9-Acridinylamino)propyl]-1,13-dioxa-4,7,10,16,19,22-hexaazacyclotetracosane (2). In a 100-mL flask, compound **7** (0.45 g, 0.29 mmol), phenol (0.6 g), and 33% HBr/AcOH (50 mL) were stirred and heated to 80 °C for 30 h. The reaction mixture was allowed to cool to room temperature, and ether (100 mL) was added; the precipitate was filtered, washed with ether (150 mL), and dried under vacuum for 4 h, affording 0.37 g of a yellow solid which was dissolved in water and basified to pH ~ 14 with 2.5 N NaOH. The free amine was extracted into CH_2Cl_2 (5 × 100 mL), and the solvent was removed. The residue was taken up in absolute EtOH and acidified to pH 1 with concentrated HCl to precipitate the desired hydrochloride salt of **2**. The solid was filtered and suspended in absolute EtOH (2 × 10 mL), and the solvent was removed before it was dried under vacuum for 48 h, affording a yellow solid (0.23 g, 90% yield): ^1H NMR (D_2O) 2.15 (brm, 2 H, $CH_2CH_2CH_2$), 4.24 (br, 2 H, CH_2NH), 7.60 (t, 2 H, aromatic), 7.84 (d, 2 H, aromatic), 8.01 (t, 2 H, aromatic), 8.46 (d, 2 H, aromatic); ^{13}C NMR 26.5 ($CH_2CH_2CH_2$), 45.4, 45.6, 46.1, 48.2, 49.4, 49.8, 50.8, 51.1 (CH_2N), 67.0, 67.3 (CH_2O), 113.7, 120.1, 125.8, 126.5, 137.2, 141.0, 159.3 (aromatic). Anal. Calcd for $C_{32}H_{63}O_2N_8Cl_8 \cdot 2H_2O$ (911.58): C, 42.16; H, 7.41; N, 12.29. Found: C, 42.14; H, 7.10; N, 11.59.

^1H NMR Studies. Spectra were recorded on a Bruker SY 200 spectrometer with solvent suppression. The chemical shifts are given in ppm with respect to *tert*-butyl alcohol as internal standard. The NMR samples (0.5 mm in 5-mm (o.d.) tubes) contained 10^{-3} M **2**·8HCl and 10^{-3} M of substrates and of competitors in D_2O adjusted to the desired pH at 25 °C using 5 N NaOH or HCl solutions. The solution pH was recorded at 25 °C by using a Metrohm 636 titrimeter and was not corrected.

Fluorescence Studies. UV/vis absorption spectra were recorded at 20 ± 0.2 °C on a Cary 219 spectrophotometer and emission spectra on a Shimadzu RF-540 spectrofluometer interfaced with a DR-3 digital recorder. The samples (3 mL in 1-cm quartz fluorescence cells) contained 10 μM **2**·8HCl or **8**, **9**, and the desired amount of substrate and competitor in distilled H_2O and 0.1 M NaOAc/AcOH buffer for experiments at pH 4 or 0.1 M Tris OAc/AcOH for pH 7.6.

Kinetic Measurements and Methods. ^{31}P NMR spectra were recorded at 80.015 MHz on a Bruker SY 200 spectrometer; the chemical shifts are given downfield from 85% H_3PO_4 as external reference. Probe temperature was regulated by the variable-temperature accessory (±5 °C). Kinetic studies were performed by following the time evolution of the proton-decoupled ^{31}P FT NMR spectra of the (substrate and macrocycle) mixtures. The NMR samples (2 mL in 10-mm (o.d.) tubes) contained 0.01 M ATP or ADP and 0.01 M macrocycle **2**·8HCl in H_2O/D_2O (9/1) adjusted to pH 7.0 ± 0.2 at 25 °C. Since the ^{31}P NMR signals of ATP, ADP, AMP, P, and the intermediate phosphoramidate **3** were distinct, the appearance or disappearance of these species could be followed simultaneously, and the relative amounts were obtained by signal integration (±5%). The values of k_{obs} calculated from the data were reproducible to ±20% at 80 °C.

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