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Macrocyclic amines as catalysts of the hydrolysis of the triphosphate bridge of the mRNA 5′-*cap* structure

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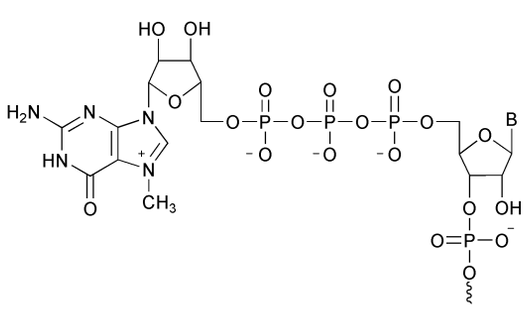
The reactions of a 5′-*cap* model compound P^1-(7-methylguanosine) P^3-guanosine 5′,5′-triphosphate, m^7GpppG, were studied in the presence of three different macrocyclic amines (**2**–**4**) under neutral conditions. The only products observed in the absence of the macrocycles resulted from the base-catalysed imidazole ring-opening and the acid-catalysed cleavage of the N^7-methylguanosine base, whereas in the presence of these catalysts hydrolysis of the triphosphate bridge predominated. The latter reaction yielded guanosine 5′-monophosphate, guanosine 5′-diphosphate, 7-methylguanosine 5′-monophosphate and 7-methylguanosine 5′-diphosphate as the initial products, indicating that both of the phosphoric anhydride bonds were cleaved. The overall catalytic activity of all three macrocycles was comparable. The hydrolysis to guanosine 5′-diphosphate and 7-methylguanosine 5′-monophosphate was slightly more favoured than the cleavage to yield guanosine 5′-monophosphate and 7-methylguanosine diphosphate. All the macrocycles also enhanced the subsequent hydrolysis of the nucleoside diphosphates, **2** being more efficient than **3** and **4**.

Introduction

The 5′-terminus of RNA polymerase II synthesised mRNA molecules contains a unique dinucleoside 5′,5′-triphosphate moiety where one of the nucleosides is N^7-methylguanosine (Scheme 1). This so called 5′-*cap* structure is required as a recognition site of various enzymes that are involved in splicing,[1–5] transport[6,7] and translation[8,9] of mRNA. The 5′-*cap* structure also protects the mRNA against intracellular exonucleases,[1,10] and viral RNA polymerases use 5′-capped mRNA sequences from the host cell as primers for the viral RNA synthesis.[11] Interactions with enzymes, and, hence, also the consequent processes, are sensitive to the correct 5′-terminal structure.[3,5,6,9,10,12] The 5′-*cap* structure is therefore a potential target site for artificial nucleases, chemical agents that sequence-selectively eliminate intracellular mRNA molecules. The principle of such catalysts is simple: an oligonucleotide recognizes a sequence of the mRNA target by hybridization, and a catalytically active function attached enhances the cleavage of the 5′-terminal *cap* structure.[13] Alternatively, the catalysts may be designed to cleave internucleosidic phosphodiester bonds,[14–18] and several oligonucleotide conjugates bearing either a metal ion complex or an organic catalyst have been shown to cleave their target RNAs selectively.



Scheme 1

The cleavage of the 5′-*cap* has been studied by using dinucleoside triphosphates as model compounds,[19–26] and it has been shown that Cu^{2+} and lanthanide ion complexes, for example, efficiently enhance the hydrolysis of the triphosphate bridge. In contrast, the other potential reactions, *viz.* the hydrolysis of the *N*-glycosidic bond of the N^7-methylguanosine moiety and the opening of the imidazole ring of the N^7-methylguanine base, are not accelerated.[26] Recent results suggest that the 5′-*cap* moiety is a promising target for artificial nucleases. It has been shown that 5′-*capped* oligonucleotides undergo selective cleavage of the triphosphate bridge when treated with a complementary oligonucleotide conjugate bearing an *N*-(2-mercaptopropionyl)glycine–Cu^{2+} complex at the 3′-terminus.[20] In addition, a 15-meric oligonucleotide conjugated with a Eu^{3+}–THED chelate has been shown to cleave the *cap* structure of an intracellular ICAM-1 RNA, significantly inhibiting the expression of the respective protein.[13]

Studies with metal ion-based catalysts have shed light on the catalysis mechanisms of the cleavage of the *cap* structure, though all details of the catalysis mechanism are not yet known. It is generally believed that a hydroxo ligand of a phosphate-bound metal ion catalyst acts as a nucleophile that attacks a phosphate group, while the coordinated metal ion electrostatically enhances the nucleophilic attack.[22–26] Alternatively, a functional group on the ligand may serve as the nucleophile.[21,25] Metal ion catalysts may, however, have a more complex role in the reaction, a metal ion aquo ligand possibly serving as a general acid-catalyst.[26]

The rate-enhancement of hydrolysis by metal ions is rather significant. A Cu^{2+}–bipyridine complex in 2 mM concentration at pH 8 and 60 °C enhances the hydrolysis of a dinucleoside triphosphate at least by a factor of 20000,[26] and various lanthanide ion complexes[25] and dinuclear Cu^{2+} complexes[22,23] appear to be even better catalysts reducing the half-life of the hydrolysis of the triphosphate to a few hours under neutral conditions and 37 °C. While efficient catalysis by metal ions has been observed, no reports on rate-enhancement by organic molecules have been published, even though they would be able to provide catalysis by the mechanisms discussed above. An organic cleaving agent, particularly if intracellular cleavage is attempted, might have advantages over the metal ion-based ones. For example, the selectivity and efficiency of metal ion-based catalysts may be reduced by coordination of the metal ion with other ligands available inside the cell. Even though no data on the cleavage of 5′-*cap* models have been reported, it is known that protonated forms of some cyclic polyamines modestly enhance the cleavage of ATP.[27–33] Under neutral conditions hexaazadioxa (**1**)[27,29] and heptaaza (**2**)[31] macrocycles are the most efficient catalysts studied. The mechanisms suggested

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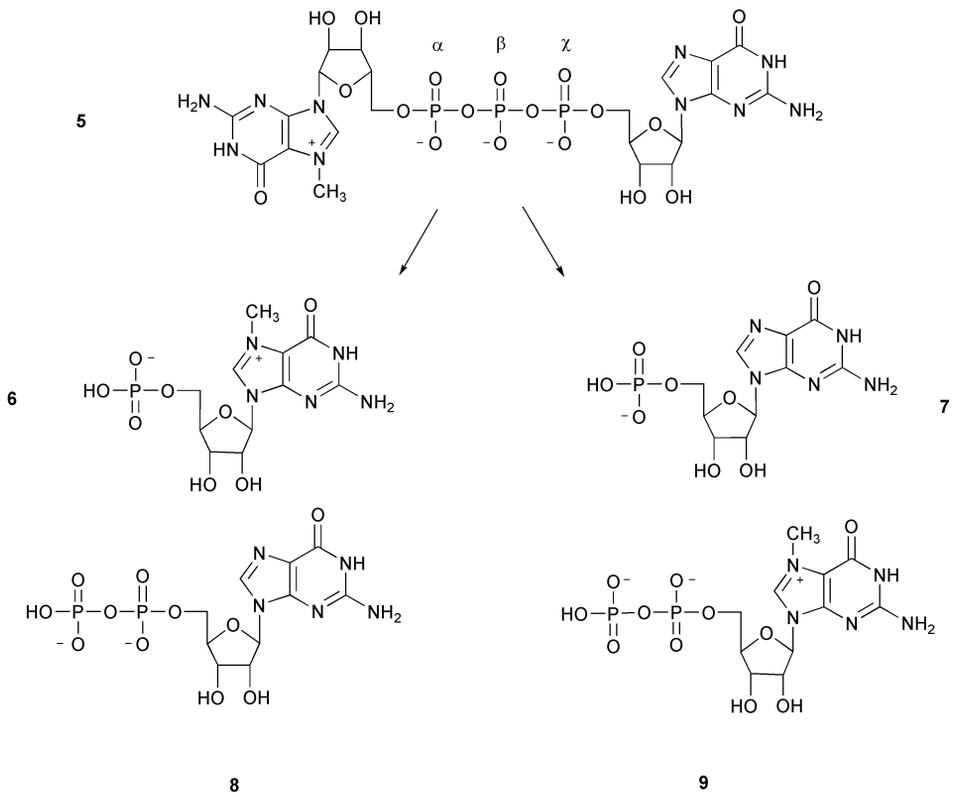
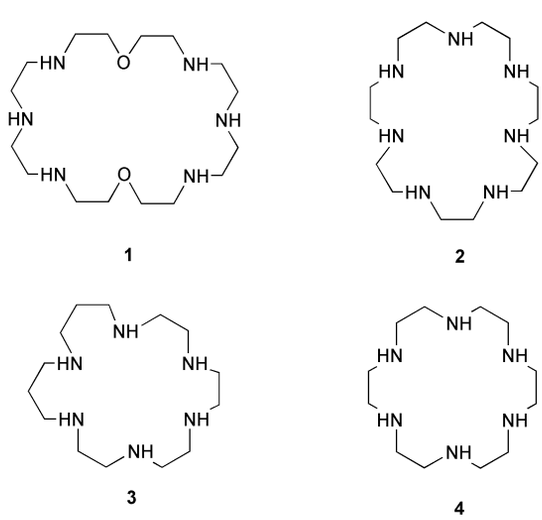


Scheme 2

for the macrocycle promoted hydrolysis of ATP are basically similar to those of metal ion promoted hydrolysis of dinucleoside triphosphates. The reaction with **1** and **2** proceeds *via* a nucleophilic attack of ring nitrogen on the phosphate group resulting in a transient formation of a phosphoramidate intermediate.[27–29,31] In cases where a phosphoramidate intermediate has not been observed, the macrocycle has been proposed to enhance the nucleophilic attack of a water molecule on the phosphate electrostatically, by hydrogen bonding or as a general acid catalyst.[30,32–34]

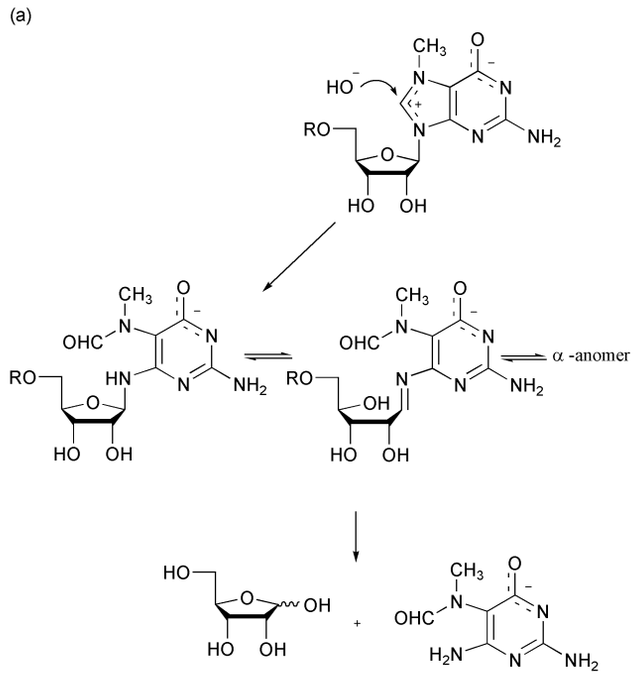


The present paper studies the effect of macrocyclic amines **2**–**4** on the hydrolysis of a 5′-*cap* model compound, P^1-(7-methylguanosine) P^3-guanosine 5′,5′-triphosphate (**5**). We show that the amines significantly enhance the hydrolysis of the triphosphate bridge to yield nucleoside monophosphate and diphosphate products (**6**–**9** in Scheme 2). To our knowledge, this is the first report on the hydrolysis of a 5′-*cap* structure promoted by purely organic catalysts.

Results and discussion

The reaction of m^{7}GpppG (**5**) in the presence of macrocyclic amines **2**–**4** was studied in 0.1 M buffer solutions at 60 °C. The concentration of m^{7}GpppG was kept below 50 μM to maintain pseudo first-order conditions. Samples taken from reaction solutions were analysed by capillary zone electrophoresis (CZE) using a 0.15 M boric acid buffer as the background electrolyte. First-order rate constants of the disappearance of m^{7}GpppG were calculated on the basis of the integrated signal areas in electropherograms. The concentrations of the more stable hydrolysis products, GMP and GDP, were determined using calibration curves obtained with commercially available compounds. The concentration of **5** in standard solutions was determined spectrophotometrically using the log ε value reported previously.[35] Samples of known concentrations were analysed using the CZE conditions mentioned above. The signal areas in the electropherograms were normalised by dividing the signal area by the migration time. Rate constants of the triphosphate hydrolysis were calculated on the basis of the increase of the concentration of the hydrolysis products as a function of time.

Under all the conditions studied, the reactions of m^{7}GpppG resulted in a complex product mixture. Of the hydrolysis products, 7-methylguanosine 5′-phosphate (m^{7}GMP; **6**), guanosine 5′-phosphate (GMP; **7**), guanosine 5′-diphosphate (GDP; **8**), and 7-methylguanosine 5′-diphosphate (m^{7}GDP; **9**) were identified by co-injecting with authentic samples. However, in addition to **6**–**9**, several other products were formed. These unknown products were difficult to identify, since several minor peaks were observed which could not be fully separated by any HPLC-method tested. Therefore, a sufficiently accurate HPLC-MS analysis or isolation of the products by chromatography were not possible. Comparison of the product mixtures obtained in the presence and in the absence of the amine catalysts shows, however, that the unknown products result from the imidazole ring-opening of the N^7-methylguanine base. This reaction takes place *via* a nucleophilic attack of a hydroxide ion on the C8 of the base, and it is known to result



Scheme 3

in a complex product mixture (Scheme 3a).[36] Products **6** and **9** undergo the ring-opening reaction slightly faster than m^{7}GpppG,[36] which further increases the complexity of the product mixture. In addition, pH-independent depurination of the 7-methylguanosine moiety yields an apurinic dimer which, owing to rapid anomerisation of the sugar moiety, may exist in two different forms (Scheme 3b).[37,38]

Fig. 1. shows the pH-dependence of the rate constant of the overall disappearance of m^{7}GpppG (k_{obs}) in the presence of macrocyclic amine **4** at 10 mM concentration. The k_{obs} values shown consist of contributions of the imidazole ring-opening (Scheme 3a), depurination of the N^7-methylguanosine moiety (Scheme 3b) and hydrolysis of the triphosphate bridge. The proportions of the three different reactions vary depending on pH. The macrocycle-promoted hydrolysis is most prominent in neutral solutions. Under these conditions hydrolysis products **6**–**9** were formed to a significant extent in the presence of the macrocyclic amines, but not at all in the absence of these catalysts. At pH > 7, the base-catalysed imidazole ring-opening[36,39,40] gradually becomes the only reaction, and above pH 8 the hydrolysis products **6**–**9** are not observed at all. Consequently, the rate increase on increasing pH refers entirely to the base-catalysed imidazole ring-opening. The macrocycle-promoted hydrolysis also becomes less significant at pH < 7. The macrocycle-dependent phosphate bridge hydrolysis is decelerated and, hence, the pH-independent depurination of

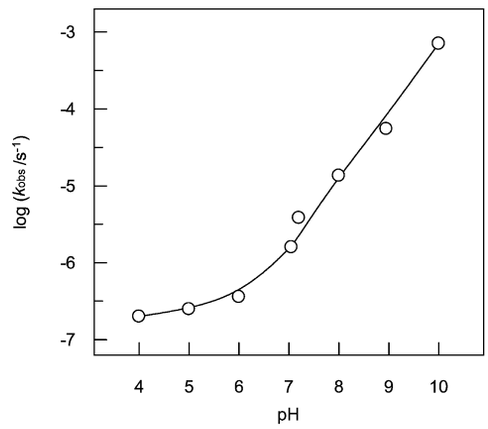


Fig. 1 The observed rate constants of disappearance of **5** (k_{obs}) as a function of pH in the presence of macrocyclic amine **4** at 60°C.

the 7-methylguanosine moiety[37,38] gradually becomes the predominant reaction, which is clearly shown by the formation of m^{7}Guo as the predominant product under slightly acidic conditions. Since the macrocycle promoted hydrolysis is observed only over a narrow pH-range, the competing reactions predominating under more acidic and basic conditions, the effect of pH on the hydrolysis could not be studied in detail.

As evidence of **5** reacting *via* the hydrolysis of the triphosphate bridge under neutral conditions, hydrolysis products **6**–**9** were formed to a significant extent in the presence of the macrocyclic amines. In the absence of the catalysts these products were not observed at all. The proportion of hydrolysis products among the product mixture was determined by calculating the concentrations of **5** and the non-methylated hydrolysis products **7** and **8**. The results of this analysis show that after 75% of **5** has reacted, the hydrolysis products constitute 70–80% of all products formed. Further evidence in favour of macrocycle catalysis was obtained by studying the effect of the amine concentration on the k_{obs} values. As is shown in Fig. 2, the rate constants increase as the concentrations of the amine catalysts increase. The hydrolysis products became clearly more predominant in the product mixture as the amine concentration increased.

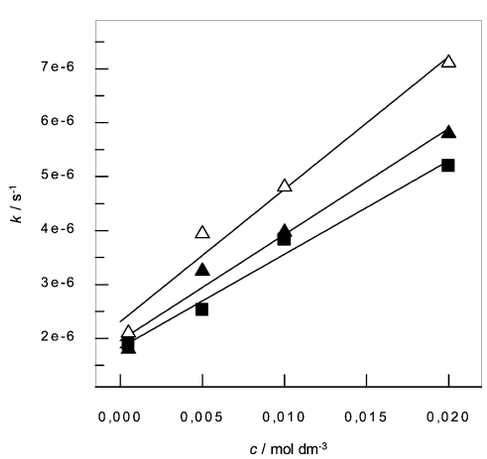


Fig. 2 The effect of concentration of **2**–**4** on the k_{obs} values of the cleavage of **5** at pH 7.2 and 60 °C. Notation: **2** – filled triangles, **3** – open triangles and **4** – filled squares.

The macrocycle-promoted hydrolysis was studied in more detail at pH 7.2. The rate constants determined in the absence of azacrowns **2**–**4** and in their 10 mM solutions are collected in Table 1. The rate constants, k_{mc}, of the macrocycle dependent reactions were obtained by subtracting from the observed rate constant (k_{obs}) the rate constant in the absence of the polyamine catalyst (k_{uncat}). In other words, azacrowns **2**–**4** are assumed

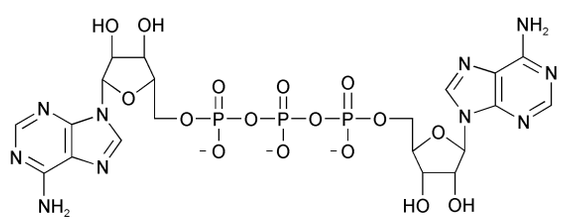
Table 1 Rate constants of the reaction of a 5′-*cap* model m7GpppG (**5**) and of nucleoside diphosphates **8** and **9** in the presence and absence of macrocyclic amines **2**–**4** at pH 7.2 and 60 °C.

	m7GpppG (**5**)	GDP (**8**)	m^7GDP (**9**)
$k_{uncat}/10^{-6}$ s^{-1}	0.97 ± 0.03		3.89 ± 0.09
k_{obs}(**2**)/10^{-6} s^{-1}	3.97 ± 0.14	36.8 ± 1.2	28.5 ± 0.8
k_{obs}(**4**)/10^{-6} s^{-1}	4.81 ± 0.19	2.50 ± 0.06	13.6 ± 0.5
k_{obs}(**4**)/10^{-6} s^{-1}	3.83 ± 0.17	1.94 ± 0.09	6.5 ± 0.4
k_{mc}(**2**)/10^{-6} s^{-1} [a]	3.0		24.7
k_{mc}(**3**)/10^{-6} s^{-1} [a]	3.8		9.8
k_{mc}(**4**)/10^{-6} s^{-1} [a]	2.9		3.6

[a] $k_{obs} - k_{uncat}$.

to accelerate only the hydrolysis of the triphosphate bridge, leaving the imidazole ring-opening of the N^7-methylguanine base and depurination of the N^7-methylguanosine moiety of **5** unaffected. This assumption appears to be valid, since the rate constants of the uncatalysed reaction (k_{uncat}) in Table 1 correspond to 20–30% of the observed k_{obs} values, which is well consistent with the proportion of hydrolysis products mentioned above. This is also consistent with previous observations that simple acyclic amines, such as triethylamine, do not promote the ring-opening reaction.[26,36]

The data in Table 1 show that azacrowns **2**–**4** at a 10 mM concentration increase the rate constants of the disappearance of m^{7}GpppG by a factor of four. The catalytic activity of all three amines is comparable, **3** being a slightly better catalyst than **2** or **4**. In the absence of **2**–**4**, the hydrolysis of m^{7}GpppG is very slow in comparison to the imidazole ring-opening. No hydrolysis products (**6**–**9**) were observed during 10 days, suggesting that the rate constant for the macrocycle-independent hydrolysis must be smaller than 5×10^{-8} s^{-1}. Evidently the reaction is even slower, since the rate constant of the uncatalysed hydrolysis of the triphosphate bridge of P^1,P^3-bis(adenosine) 5′,5′-triphosphate (**10**) has been estimated to be less than 5×10^{-9} s^{-1} at 60 °C.[26] Accordingly, macrocyclic amines **2**–**4** at a 10 mM concentration appear to enhance the hydrolysis of m^{7}GpppG by a factor of a few hundred.



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The hydrolysis of the triphosphate bridge of **5**, in principle, results in the formation of four different products (Scheme 2). Cleavage of a bond between α and β phosphates would result in the formation of m^{7}GMP and GDP, while cleavage between β and χ phosphates would yield m^{7}GDP and GMP. The predominant hydrolysis products observed however varied depending on the catalyst employed. This results partly from the fact that the ring-opening reaction of the N^7-methylated products is faster than that of **5**. Furthermore, in some cases the macrocyclic amines enhance the cleavage of the products as well. The effect of **2**–**4** on the hydrolysis products was studied by independent experiments, and the rate constants obtained with nucleoside diphosphates **8** and **9** are included in Table 1.

With **3** and **4** as the catalyst of the cleavage of **5**, the predominant products observed were GMP (**7**) and GDP (**8**), the amounts of m^{7}GMP (**6**) and m^{7}GDP (**9**) were clearly smaller, particularly for longer reaction times. Both amines enhance the hydrolysis of m^{7}GDP and **3** also slightly enhanced the disappearance of m^{7}GMP. There is no doubt that **3** and **4** promote the cleavage between both α and β, and β and γ phosphates (Scheme 2). The cleavage between the α and β phosphates is slightly favoured in both cases: after 75% of **5** has reacted, the GDP : GMP concentration ratios were approximately 2 : 1 and 3 : 1 with amines **3** and **4**, respectively. In the presence of **2** the situation is a bit more complicated, since the catalyst also efficiently promoted the hydrolysis of GDP to GMP, and the only product observed to any significant extent was GMP. Most probably, however, **2** also enhances the hydrolysis of **5** between α and β phosphates as well as between β and χ phosphates, but the proportion of the two reaction routes cannot be determined on the basis of the data available.

As was mentioned in the introduction, several mechanistic alternatives have been proposed for the macrocycle-promoted hydrolysis of ATP. These include nucleophilic catalysis by unprotonated ring nitrogens, facilitation of nucleophilic attack on phosphorus by electrostatic interaction or hydrogen bonding of the phosphate oxygens with the protonated ring nitrogens and general acid/base catalysed attack of a water molecule. The same mechanisms may also operate in the present case. The species distribution curves in Fig. 3, based on reported pK_a values,[41,42] show that the predominant species of the heptaamine **2** and hexamine **4** at pH 7.2 at 60 °C is a trication. Such a structure, containing both protonated and unprotonated amines, could, in principle, catalyse the hydrolysis of the triphosphate bridge by any of the mechanisms proposed. An MS analysis of a sample from the reaction solution was carried out to detect a possible phosphoramidate intermediate, but without success. This cannot, however, be taken as conclusive evidence against the mechanism where the attacking nucleophile is ring nitrogen since the intermediate may be too short-lived to accumulate. On using ATP as a substrate, the phoshoramidate was only observed with the most efficient polyamine catalysts, **1** and **2**. Even though relatively high concentrations of the reactants were used, the decomposition of the intermediate generally was faster than its formation.[28] The fact that the catalytic efficiency of azacrowns **2**–**4** is highest when approx-

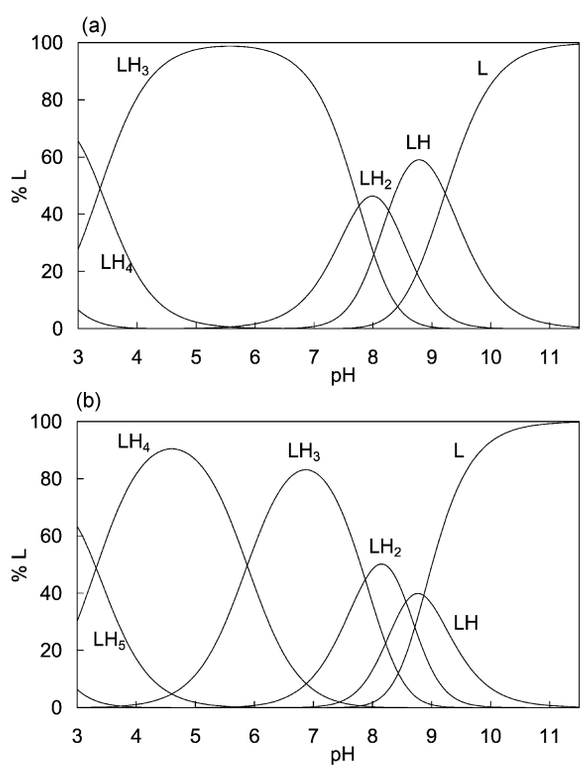

Fig. 3 a. Species distribution of macrocyclic amine **2** at 60 °C. pK_a values taken from ref. 41 have been extrapolated using the temperature dependence of pK_a values of **4** reported in ref. 42. b. Species distribution of macrocyclic amine **4** at 60 °C. pK_a values at 60 °C have been extrapolated using the temperature dependence reported in ref. 42.

imately half of the nitrogen atoms are protonated could well be accounted for by a mechanism involving an attack of unprotonated ring nitrogen on phosphorus assisted by interaction of the phosphate oxygens with the positively charged ammonium ion centers on the ring.

Polyamines **2**–**4** show interesting selectivity that cannot be explained by the present data. Even though **2**–**4** hydrolyse m^7GpppG approximately equally efficiently, their abilities to hydrolyse m^7GDP and GDP differ considerably, **2** being the most and **4** the least effective catalyst. Comparison of the present data with those reported for ATP shows that the effect of cyclic polyamines on the hydrolysis of the *cap* structure cannot be predicted by their influence on the hydrolytic stability of ATP. For example, even though **2** and **4** are equally efficient catalysts of the hydrolysis of m^7GpppG, only **2** significantly hydrolyses ATP.[31] Another indication of the dissimilarity of the two systems is that **1**, which is a good catalyst of ATP hydrolysis,[27–29] does not enhance the hydrolysis of a 5′-*cap* model **10**.[26] In contrast, a $2Cu^{2+}$–**1** complex efficiently catalyses the hydrolysis of **10**,[26] whereas complex formation does not affect the catalysis of ATP hydrolysis.[29]

Experimental

Materials

Guanosine 5′-diphosphate and guanosine 5′-monophosphate were products of Sigma and they were used as received. 1,4,7,10,13,16-Hexaazacycloicosane trisulfate (Sigma) was transferred to the free base eluting through Dowex (OH^-) resin. N^7-Methylguanosine, N^7-methylguanosine 5′-monophosphate and N^7-methylguanosine 5′-diphosphate were prepared using methyl iodide as methylating reagent as described before.[43] m^7GpppG was synthesised according to a literature method [36,44] starting from guanosine 5′-phosphorimidazolide and 7-methylated guanosine diphosphate. The coupling reaction was carried out in the presence of $ZnCl_2$ in anhydrous DMF. The compounds were characterized by ^{1}H and ^{31}P NMR and EI-MS. The spectral data obtained are consistent with those reported in the literature. ^{1}H NMR (D_2O, 400 MHz): 7.84 (s, 1H), 5.72 (d, 3.0 Hz, 1H), 5.62 (d, 5.96 Hz, 1H), 4.48 (t, 5.56 Hz, 1H), 4.34 (t, 3.2 Hz, 1H), 4.06–4.32 (m, 8H), 3.87 (s, 3H). ^{31}P NMR (D_2O, 161.7 MHz): −11.24 (d, 17.8 Hz, Pa and Pr), −22.74 (t, 20.20 Hz, Pb). Mass: 805.1 (M + 1), 400.4 (M/2).

Synthesis of macrocyclic amines 2 and 3

2 And **3** were prepared according to a modification of the Richman–Atkins cyclisation method using caesium carbonate as a base.[45,46] Fully protected macrocycles were obtained by coupling the bis(2-mesyloxyethyl) derivative of diethylenetriamine with fully tosylated triethylenetetramine hydrate or *N*-(3-aminopropyl)-1,3-propanediamine. After HAc/HBr/phenol deprotection of the tosyl groups, the HBr salts were transferred to the free base by eluting through Dowex (OH^-) resin. The products were characterized by ^{1}H NMR and ^{13}C NMR and mass spectra. 1,4,7,10,13,16,19-Heptaazacycloeicosane (**2**): ^{1}H NMR (D_2O, 400 MHz): 3.582 (s, 28H). ^{13}C NMR (D_2O, 400 MHz): 43.789. Mass: 302.1 (M + 1). 1,4,7,10,13,17-Hexaazacycloeicosane (**3**): ^{1}H NMR (D_2O, 400 Hz): 1.4994 (m, 4H), 2.454 (m, 9H), 2.5063 (m, 15H); ^{13}C NMR (D_2O, 400 Hz): 48.168, 48.115, 47.963, 47.819, 46.460, 46.187; HRMS: 287.292320 (found), 287.292300 (calculated for $C_{12}N_6H_{30}$).

Preparation of reaction solutions

The pH of the reaction solutions was adjusted with 0.1 M acetic acid, MES (morpholino ethanesulfonic acid; pK_a 6.2 at 25 °C), MOBS (4-[*N*-morpholine] butanesulfonic acid; pK_a =7.6 at 25 °C), HEPES (2-[4-(2-hydroxyethyl)-1-piperazino]ethanesulfonic acid; pK_a = 7.5 at 25 °C), or CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid, pK_a = 9.3 at 25 °C) buffer. The pH of the reaction solutions was checked with a pH-meter at 25 °C. The ionic strength was adjusted to 0.1 M with $NaNO_3$.

Kinetic measurements

Reactions were carried out in Eppendorf tubes immersed in a water bath, the temperature of which was thermostated at 60 °C. The reactions were initiated by adding substrate stock solution (a few microliters) to give a concentration of 50 μM. The total reaction volume was 1.5 ml. Aliquots (12) of 100 μl were withdrawn at appropriate intervals to cover approximately two half-lives of the reaction. The aliquots were immediately cooled down over an ice-bath, and stored in the freezer until analysed.

Analysis of aliquots

Aliquots were analysed by capillary zone electrophoresis (CZE). Capillary electrophoretic analysis was carried out in a fused silica capillary (75 μm id, 110 cm) with 0.15 M boric acid, pH 8.5 as the run buffer. A voltage of 30 kV was applied. The compounds were detected at 254 nm. Under these conditions the migration time of the starting material was about 60 min.

Acknowledgements

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