Interactions between aminocalixarenes and nucleotides or nucleic acids †

Youheng Shi and Hans-Jörg Schneider *

FR Organische Chemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany

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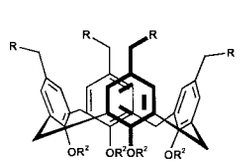
Four calixarenes with (trimethylammonium)methyl groups at the phenyl rings in the upper rim were prepared. Association constants K with mononucleotides were determined in D_2O by NMR shift titration, partially also by fluorescence competition titration using ANS as dye. The complexation free energies ΔG obtained with the derivatives of the calix[4]cone (**AC4c**) and the calix[4]-1,3-alternate (**AC4a**) conformation were similar, but increased from AMP (18 ± 1 kJ mol^{-1}) to ADP (20 ± 1 kJ mol^{-1}), to ATP (22 ± 1 kJ mol^{-1}). With the calix[6] derivative (**AC6**) the corresponding values were 22, 24, 27 kJ mol^{-1}, with the calix[8] host (**AC8**) 24, 26, 28 kJ mol^{-1}, respectively. The large contribution of salt bridging to the complexation was obvious from the ΔG difference between adenosine and *e.g.* AMP (with the calix[4]cone derivative 5.6 and 17.7 kJ mol^{-1}, respectively). Affinity differences between different nucleobases increased moderately with the size of the macrocyclic host, *e.g.* $\Delta\Delta G$ between AMP and TMP was 1 kJ mol^{-1} with calix[4]cone, 2 kJ mol^{-1} with calix[6], and 3 kJ mol^{-1} with calix[8] compounds. The results are in line with computer simulated complex structures in which the nucleobase or sugar parts are only partially inserted into the calix cavity. This agrees with the observed complexation induced NMR shifts (CIS), which are small but increase with the ring size of the host. Noticeably the CIS values are substantially larger for much weaker bound nucleosides.

Affinities of the four aminocalixarenes with double-stranded calf thymus (CT) DNA, with polydA*polydT and with polydG*polydC were characterized by ΔT_m of the double-strand denaturation temperature and by fluorimetric assays using ethidium bromide (C_{50} values). The calix[4]cone derivative **AC4c** shows, due to the four positive charges converging at one side, the strongest effects. They surpass spermine although this also bears four protonated ammonium groups, indicating additional binding contributions from the phenyl moieties. The larger, more flexible calix[6]- and calix[8]-derivatives **AC6** and **AC8** show only small affinity increases in spite of their 6 or 8 positive charges. Preliminary molecular modeling studies indicate that based on the distances between the ammonium centers only partial contact of all centers to the groove phosphates can materialize. The ligands **AC4c**, **AC4a** and **AC6** exhibit a remarkable preference for DNA in comparison to RNA mimics.

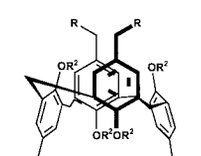
Aims and introduction

Molecular recognition of nucleotides by synthetic receptors [1–11] and small ligand interactions with nucleic acids [12,13] have recently received much attention. Macrocyclic polyamines, when protonated, bind strongly to nucleotides *via* electrostatic interactions and/or hydrogen bonding between the cationic ammonium groups of the receptor and the negatively charged nucleotide phosphate groups.[2,4] Previously we used tetraazonia cyclophanes,[11a,b] aminocyclodextrins [11c] and hexaazonia cyclophanes [11d] for achieving binding constants K of up to 10^5 M^{-1}, and selectivities, *e.g.* between nucleobases, between 3′- or 5′-substitution sites, and between 2′-oxy/deoxynucleotides with corresponding K differences of up to an order of magnitude.

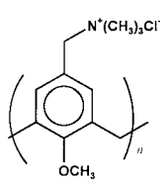
In the present work we explore the application of calixarenes, which to the best of our knowledge have until now not been used for nucleotide complexation or as ligands for DNA. These macrocyclic polyphenol derivatives allow the introduction of a variety of desired binding functions into molecular receptors of designed flexibility and shape.[14–18] As with other synthetic host systems (see. *e.g.*, ref. 11*c*) one may, in addition to the anion binding sites, introduce other sites capable of interactions with the sugar moiety and/or the nucleobase. Interactions with the nucleobase may be achieved either by stacking with aromatic parts, or by sites capable of forming complementary hydrogen-bonding patterns. We have prepared calixarenes with four, six or eight permethylammonium moieties at the upper rim; the permanently charged groups provide both for water-solubility and for pH-independent anion binding sites. These aminocalixarenes (**AC**, Scheme 1) can in addition interact with the sugar and in particular with the nucleobase moieties if their geometry allows contact with other more lipophilic parts of the macro-



AC4c (R = $N^+(CH_3)_3$ Cl^-, R^2 = C_3H_7)



AC4a (R = $N^+(CH_3)_3Cl^-$, R^2 = CH_3)



AC6 $n = 6$
AC8 $n = 8$

Scheme 1

† NMR data and elemental analyses as well as Tables 9–12 (containing NMR shifts, complexation-induced shifts, equilibrium constants and binding free energies) are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/1999/1797, otherwise available from BLDSC (SUPPL. NO. 57563, pp. 3) or the RSC Library. See Instructions for Authors available *via* the RSC web page (http://www.rsc.org/authors).

cycles. The larger **AC** derivatives also hold promise as molecular containers for selective transport *e.g.* to DNA or RNA of other effectors bound in the cavity.[19]

Experimental

Materials

5,11,17,23-Tetrakis(trimethylammoniomethyl)-25,26,27,28-tetramethoxycalix[4]arene tetrachloride (**AC4a**), 5,11,17,23-tetrakis(trimethylammoniomethyl)-25,26,27,28-tetrapropoxycalix[4]arene tetrachloride (**AC4c**), 5,11,17,23,29,35-hexakis-(chloromethyl)-37,38,39,40,41,42-hexamethoxycalix[6]arene (**C6**) and 5,11,17,23,29,35,41,47-octakis(chloromethyl)-49,50,51,52,53,54,55,56-octamethoxycalix[8]arene (**C8**) were synthesized according to the literature.[15,16]

AC6, AC8. In 25 ml of DMF containing **C6** (0.20 g) or **C8** was introduced trimethylamine (gas) for 2–3 hours at room temperature. The precipitate was collected by filtration, washed with chloroform, and dried *in vacuo* to give 5,11,17,23,29,35-hexakis(trimethylammoniomethyl)-37,38,39,40,41,42-hexamethoxycalix[6]arene hexachloride (**AC6**) (yield: 35%), and 5,11,17,23,29,35,41,47-octakis(trimethylammoniomethyl)-49,50,51,52,53,54,55,56-octamethoxycalix[8]arene octachloride (**AC8**) (yield: 20%). All compounds decomposed before melting.

Spectroscopy and calculations

NMR studies were performed with a Bruker AM-400 system in D_2O using TMS/CCl_4 as an external standard at 298 ± 1 K. NMR Data and elemental analyses are given in the Supplementary Data.† Proton chemical shifts were reproducible to within 0.001 ppm. Binding constants and complexation-induced shift (CIS) values were determined as before[11b] from nonlinear least-squares fits.

Fluorescence binding titration was carried out with a HITACHI F-2000 fluorescence spectrophotometer, using in competition experiments 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe. After determination of the binding constants K for ANS with the different calixarenes the K values for nucleotides were obtained as described in the literature.[20] Preliminary molecular modeling studies were performed under gas phase conditions ($\varepsilon = 2$ Debye) using QUANTA/CHARMm (version 3.3 from MSI).[21]

DNA Studies

Calf thymus DNA, polydA*polydT and polydG*polydC were purchased from Sigma and used after sonification without further purification.

Binding experiments. Binding experiments with ethidium bromide were carried out in analogy to the literature.[23] Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridium bromide, 1.26 μM final concentration) was dissolved in 2 ml of SHE (sodium 4-(2-hydroxyethyl)piperazine-1-ethanesulfonate) buffer (SHE buffer of ionic strength 0.01 contained 2 mM of HEPES *N′*-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid, 10 μM of EDTA, and 9.4 mM of NaCl). The pH was adjusted to 7.0 with NaOH. A solution of DNA was added to reach a concentration of 3.6 μM in base pair. The fluorescence was recorded on a Hitachi F-2000 fluorescence spectrophotometer with emission and excitation wavelengths at 595 nm and 546 nm, respectively. Aminocalixarene aqueous solution was added to the DNA–ethidium solution in microliter portions with Eppendorf pipettes, and the fluorescence decrease was measured after each addition until more than a 50% reduction of fluorescence was observed. The C_{50} value, defined as the aminocalixarene concentration required to reduce the fluorescence of the DNA–ethidium complex to 50% was obtained from graphical evaluation of the curves. All measurements were made in triplicate. Error limits on C_{50} values are ±10%.

Thermal denaturation measurements

Thermal denaturation measurements were conducted with a Cary 1 Bio UV-Visible spectrophotometer interfaced to a PC by following the absorption change at 260 nm as a function of temperature. The temperature was controlled by a Cary temperature controller programmed to raise the temperature at a rate of 1.0 °C min^{-1}. A thermistor fixed into a reference cuvette was used to monitor the temperature. Denaturation experiments were carried out in SHE buffers (0.01 M). T_m values were determined from the first-derivative plots and were obtained at several aminocalixarene/nucleic acid phosphate ratios r. All measurements were made in duplicate. Error limits on ΔT_m values are ±0.5 °C.

Results and discussion

NMR Characterization of the compounds

The aminocalix[4]arenes **AC4a** and **AC4c** (Scheme 1), prepared according to literature procedures,[15] were used as tetrahydrochloride salts, as were the larger aminocalix[6]arene (**AC6**) and aminocalix[8]arene (**AC8**), synthesized from the known[15,16] calixarenes **C6** and **C8** by reaction with trimethylamine. It is known that in tetra-*O*-alkylcalix[4]arenes the methyl group is too small to suppress the oxygen-through-the annulus rotation.[14a] The ^{1}H NMR spectra of **AC4a** in D_2O showed a CH_2 singlet at 4.24 ppm and only one aromatic peak at 7.16 ppm, indicating the symmetry of the 1,3-*alternate* conformation. The introduction of four *n*-propyl groups, bulkier than ethyl, at the lower rim of calix[4]arenes locks the macrocycle in one of the four possible stereoisomeric configurations: *cone, partial cone*, 1,3-*alternate*, 1,2-*alternate*.[14a] The stereochemical result of the synthesis is highly dependent on the nature of the base, countercation, solvent and temperature. The conditions used to obtain the *cone* isomer **AC4c** here involved the use of NaH in DMF at room temperature. Two double peaks at 4.38 and 3.22 ppm were observed in the ^{1}H NMR spectrum of **AC4c** in D_2O, which confirms that only the *cone* isomer was present.

Due to the larger dimension of the macrocycle the calix[6]- and calix[8]arenes form conformationally mobile derivatives. The ^{1}H NMR spectrum of **AC6** with a multiplet for the methylene bridge ArCH_2Ar (δ 3.76 ppm) suggest that the conformation is partially cone. Preliminary molecular mechanics calculations with the CHARMm[21] force field suggest that the cone conformation has the lowest energy. Due to the electrostatic repulsions between the permethyl ammonium groups, **AC8** is conformationally more mobile and its ^{1}H NMR spectrum in D_2O shows a broad singlet for the methylene bridge ArCH_2Ar (δ 3.76 ppm).

Interactions with nucleosides and nucleotides

Representative ^{1}H NMR titration curves for the complex of AMP–**AC6** are illustrated in Fig. 1. The ^{1}H NMR spectra of the nucleotides and nucleosides show that the proton signals of both the nucleobase and of the sugar are shifted upfield upon complexation with the cyclophane (Tables 1 to 6 and 10, see supplementary data). Complexation-induced chemical shifts (CIS values), obtained from the non-linear least square fit, vary from −0.01 ppm to −0.37 ppm shielding and indicate in all cases that the nucleotides are only partially inserted within the cavity of calixarene, in contrast to a nucleotide–cyclophane system,[11b] to a sulfonate calix[4]arene–ethanol complexes,[17] and to calix[5]arene–alkylammonium complexes,[18] where the guests show larger upfield shifts, typical for intracavity inclusion within the cyclophane.

Table 1 NMR Shifts (δ_0), complexation-induced shifts (CIS), equilibrium constants (K, M^{-1}) and binding free energy ($-\Delta G$, kj mol^{-1}) of adenine derivatives with **AC4a**[a]

Compound		H-8	H-2	H-1′	K_{av} and ΔG_{av}
AMP^{2-}	δ_0	8.42	8.08	5.96	
	CIS	−0.012	−0.050	−0.051	
	$K \times 10^{-3}$	2.4 ± 0.2	1.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.2
	ΔG	19.2	18.3	18.8	18.8
ADP^{3-}	δ_0	8.34	8.07	5.95	
	CIS	−0.035	−0.088	−0.099	
	$K \times 10^{-3}$	3.1 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	2.6 ± 0.2
	ΔG	19.9	19.1	19.2	19.5
ATP^{4-}	δ_0	8.36	8.08	5.96	
	CIS	−0.033	−0.103	−0.111	
	$K \times 10^{-3}$	4.9 ± 0.7	7.0 ± 0.9	7.1 ± 0.9	6.7 ± 0.9
	ΔG	21.0	21.9	22.0	21.8

[a] Measured at 298 ± 1 K in D_2O; all shifts in ppm (±0.005). K is in mol^{-1} (±20%), unless noted otherwise. CIS from nonlinear least-squares fit where single K values are given; in other cases CIS are obtained from single measurements at selected concentrations, calculated with K values from fluorescence titrations.

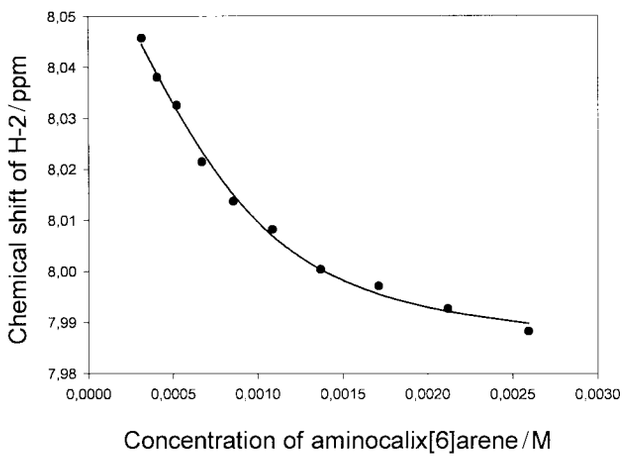


Fig. 1 Example of NMR shift titration (**AC6** with AMP, conditions see Experimental section).

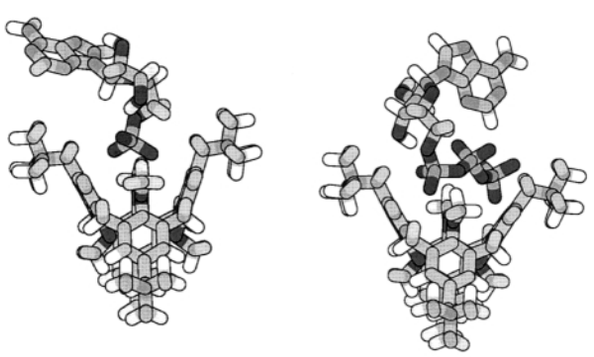


Fig. 2 QUANTA/CHARMm energy minimized structures of the complexes of **AC4a**–ADP and **AC4a**–ATP.

The binding isotherms in the NMR titrations usually gave satisfactory fits for 1:1 complexes (if $K \leq 2 \times 10^4$ M^{-1}). For binding constants above 10^4 M^{-1} it is difficult to obtain satisfactory binding isotherms by NMR spectroscopy as a consequence of the limited NMR sensitivity. Therefore, and as complexation did also not produce significant UV or fluorescence changes the stability constants of stronger complexes were measured mostly by competitive fluorescence spectrophotometry. The values obtained by the fluorometric competition procedure were highly reproducible (average deviation $\Delta K < 10\%$), and agreed within ±20% with the K values obtained by NMR titration, wherever such a comparison was possible.

Tables 1–6 (also Tables 9 and 10, see supplementary data) show that the binding constants of all nucleotide monophosphates with **AC4c** or **AC6**, or **AC8** differ to some degree, but are in general of the same order. The strong electrostatic interaction between the ionic groups enforces an orientation with diminished contact between the nucleobase part and the aryl parts of the **AC**, resulting in less nucleobase selectivity. This is also in line with preliminary molecular mechanics calculations. It is known that each salt bridge in water is worth up to 5 ± 1 kJ mol^{-1} for electrostatic host–guest binding if the conformations allow sufficient contact between the anion and cation.[11a,b,d,22] By applying this value to the complexation of nucleobase monophosphate with **AC4** one can conclude that only two salt bridges materialize in the complex. That strain induced upon complexation reduces the affinity is less likely: force field energy minimizations indicate that *e.g.* for the **AC4a** system the distances d between Me_3N^+–––$^+NMe_3$ groups at the 1,3-position at the AMP binding site are 12.6 Å before, and 12.0 Å after complexation; the distances at the lower part not involved in the association remain constant at d = 12.0 Å.

The binding free energies (ΔG) increase by only about 2 kJ mol^{-1} from mono- to diphosphate, and by 3–4 kJ mol^{-1} from di- to triphosphate. Noticeably, ΔG for AMP increases by 3–4 kJ mol^{-1} from **AC4c** to **AC6**, and to **AC8** pointing to nucleobase interactions with the calixarene aryl residues. In line with computer aided molecular modeling the larger macrocycles allow deeper, although still incomplete insertion of the guest molecule into the cavity. The same order was also observed in the complexation free energy of ANS dye, which increases from ANS–**AC4a** (ΔG = 19.6 kJ mol^{-1}), to ANS–**AC6** (ΔG = 21.1 kJ mol^{-1}), and to ANS–**AC8** (ΔG = 24.7 kJ mol^{-1}).

If one subtracts the ΔG values observed for electroneutral nucleosides from derivatives containing 2 negative charges, one obtains 5 ± 1 kJ mol^{-1} for one ion pair or so-called salt bridge (Tables 1–6, 9 and 10 of the supplementary data), in gratifying agreement with earlier general values.[11a,b,22] This value becomes smaller if the geometry does not allow optimal contact between host and guest charge with simultaneous preservation of another two anion–cation optimal contacts, as is obvious from computer aided modeling. Remarkably, the CIS values on the nucleosides in spite of the weaker complexes are *larger* than on the nucleotides, indicating that the strong electrostatic interaction hinders the interaction between the nucleobases and the aminocalixarenes.

Molecular modeling secures the geometric boundaries for sufficient salt bridges in the complexes. The distance between opposite N^+ in the 1,3-alternate conformation (**AC4a**) is 10 to 12 Å (see above). The distance between opposite and neighbor N^+ groups in the cone conformation (**AC4c**) is 12 Å and 9 Å, respectively. In **AC6** the distance between the N^+ groups in A/D, in A/C, and in A/B aryl ring position is 15 Å, 10–14 Å, 8 Å,

Table 2 NMR Shifts (δ_0), complexation-induced shifts (CIS), and equilibrium constants (K/M^{-1}) and binding free energy ($-\Delta G$/kJ mol^{-1}) of adenine derivatives with **AC4c**[a]

Compound		H-8	H-2	H-1′	K_{av} and ΔG_{av}
adenosine	δ_0	8.13	8.03	5.86	
	CIS	−0.27	−0.37	−0.22	
	K	9	10	9.6	9.6
	ΔG	5.4	5.7	5.6	5.6
AMP^{2-}	δ_0	8.42	8.08	5.96	
	CIS	−0.034	−0.058	−0.050	
	$K \times 10^{-3}$	2.0 ± 0.4	0.92 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
	ΔG	18.8	16.9	17.3	17.7
ADP^{3-}	δ_0	8.35	8.08	5.96	
	CIS	−0.071	−0.080	−0.096	
	$K \times 10^{-3}$	3.4 ± 0.8	1.6 ± 0.2	3.1 ± 0.6	2.7 ± 0.5
	ΔG	20.1	18.3	19.9	19.6
ATP^{4-}	δ_0	8.36	8.08	5.96	
	CIS	−0.085	−0.072	−0.102	
	$K \times 10^{-3}$	6.4 ± 3.1	12 ± 5.6	14 ± 5.6	11 ± 4.8
	ΔG	21.7	23.2	23.6	23.0

[a] See footnote to Table 1.

Table 3 NMR Shifts (δ_0), complexation-induced shifts (CIS), and equilibrium constants (K/M^{-1}) and binding free energy ($-\Delta G$/kJ mol^{-1}) of adenine derivatives with **AC6**[a]

Compound		H-8	H-2	H-1′	K_{av} and ΔG_{av}
AMP^{2-}	δ_0	8.42	8.08	5.96	
	CIS	−0.053	−0.095	−0.090	
	$K \times 10^{-3}$	10.0 ± 2.0	5.2 ± 0.8	4.2 ± 0.5	6.5 ± 1.1
	ΔG	22.8	21.2	20.7	21.7
ADP^{3-}	δ_0	8.36	8.08	5.96	
	CIS	−0.052	−0.098	−0.150	
	$K \times 10^{-3}$	6.6 ± 1.9	18 ± 5.6	14 ± 3.1	12.9 ± 3.8
	ΔG	21.8	24.2	23.6	23.5
ATP^{4-}	δ_0	8.36	8.08	5.96	
	CIS	−0.063	−0.115	−0.132	
	$K \times 10^{-3}$	60 ± 30	63 ± 30	72 ± 32	65 ± 31
	ΔG	27.2	27.4	27.7	27.4

[a] See footnote to Table 1.

Table 4 NMR Shifts (δ_0), complexation-induced shifts (CIS), and equilibrium constants (K/M^{-1}) and binding free energy ($-\Delta G$/kJ mol^{-1}) of adenine derivatives with **AC8**[a]

Compound		H-8	H-2	H-1′	K_{av} and ΔG_{av}
AMP^{2-}	δ_0	8.42	8.08	5.96	
	CIS	−0.042	−0.10	−0.084	
	$K \times 10^{-3}$	15 ± 5	11 ± 4	13 ± 2.4	13 ± 4
	ΔG	23.8	23.0	23.4	23.4
ADP^{3-}	δ_0	8.36	8.08	5.96	
	CIS	−0.049	−0.084	−0.140	
	$K \times 10^{-3}$	43 ± 20	34 ± 14	45 ± 15	40 ± 15
	ΔG	26.4	25.8	26.5	26.1
ATP^{4-}	δ_0	8.36	8.08	5.96	
	CIS	−0.137	−0.137	−0.137	
	$K \times 10^{-3}$	76 ± 34	55 ± 24	68 ± 30	66 ± 30
	ΔG	27.8	27.0	27.5	27.5

[a] See footnote to Table 1.

respectively. In **AC8** these distances are 18 to 20 Å (A/D), 12 to18 Å (A/C); and 7 to 9 Å (A/B), respectively. The computer simulations suggest that in the complexes of ADP and ATP with aminocalixarenes the small increase of ΔG is the consequence of the rather poor geometric fit and the nonideal contact between the third (or the fourth) anion and cation in this case. Comparison of **AC4a** or **AC4c** with **AC6** and **AC8** in their association behavior towards the AMP clearly indicate that van der Waals effects contribute significantly. The binding increase generated by the nucleobase finds its explanation in the possible approach of this nucleobase to the N^+ atom of the larger aminocalixarenes.

Interactions with DNA

The binding of the new aminocalixarenes must be seen in the context of polyamines, which usually show a rather linear dependence of binding affinity to double-stranded DNA on the number of positive charges in the ligand.[23] This can be rationalized by the number of salt bridges between the nitrogen centers and the nucleotide phosphate groups, with an average value of 5 kJ mol^{-1} for one ion pair.[11a,b,22] Due to counterion and salt effects,[12a] respectively, this value was recently[24] found to be as large as 8 kJ mol^{-1} if extrapolated to zero ionic strength. The presence of other structural fragments such as aromatic

Table 5 NMR Shifts (δ_0), complexation-induced shifts (CIS), and equilibrium constants (K/M^{-1}) and binding free energy (ΔG/kJ mol^{-1}) of selected nucleotides with **AC4a**[a]

Compound		H-5	H-6	H-8	CH_3	H-1′	H-2′	H-3′	H-4′	H-5′	K_{av} and ΔG_{av}
GMP^{2-}	δ_0			8.00		5.75		4.30	4.13	3.82	
	CIS			−0.01		−0.052					
	$K \times 10^{-3}$					0.8 ± 0.08					0.8 ± 0.08
	ΔG					16.5					16.5
UMP^{2-}	δ_0	7.94	5.82			5.80	4.24	4.17	4.07	3.83	
	CIS	−0.02	−0.030			−0.021					
	$K \times 10^{-3}$	0.63 ± 0.1	0.85 ± 0.1			1.1 ± 0.1					0.85 ± 0.1
	ΔG	16.0	16.7			17.3					16.7
CMP^{2-}	δ_0	5.95	7.92			5.82	4.16	4.16	4.06	3.86	
	CIS	−0.032	−0.031			−0.040					
	$K \times 10^{-3}$	0.73 ± 0.0 8	0.62 ± 0.0 6			0.73 ± 0.0 6					0.70 ± 0.07
	ΔG	16.3	15.9			16.3					16.2
TMP^{2-}	δ_0		7.62		1.74	6.16		4.39	3.96	3.80	
	CIS		−0.026		−0.061	−0.090				−0.020	
	$K \times 10^{-3}$		0.42 ± 0.1		0.35 ± 0.0 4	0.35 ± 0.0 4				0.72 ± 0.1 5	0.37 ± 0.07
	ΔG		15.0		14.5	14.5				16.2	15.0

[a] See footnote to Table 1. [b] CIS and K values not accessible due to too small shifts or to masked signals. [c] Signal masked by HDO peak. GMP = guanosine 5′-phosphate, UMP = uridine 5′-phosphate, CMP = cytidine 5′-phosphate, TMP = thymidine 5′-phosphate.

Table 6 NMR Shifts (δ_0), complexation-induced shifts (CIS), and equilibrium constants (K/M^{-1}) and binding free energy (ΔG/kJ mol^{-1}) of selected nucleotides with **AC4c**[a]

Compound		H-5	H-6	H-8	CH_3	H-1′	K_{av} and ΔG_{av}
GMP^{2-}	δ_0			8.00		5.75	
	CIS					−0.071	
	$K \times 10^{-3}$					0.31 ± 0.06	0.31 ± 0.06
	ΔG					14.2	14.2
UMP^{2-}	δ_0	7.94	5.82			5.80	
	CIS	−0.032	−0.034			−0.060	
	$K \times 10^{-3}$	0.74 ± 0.1	0.35 ± 0.1			0.42 ± 0.08	0.47 ± 0.1
	ΔG	16.3	14.5			15.0	15.2
CMP^{2-}	δ_0	5.95	7.92			5.82	
	CIS	−0.045	−0.032			−0.046	
	$K \times 10^{-3}$	1.2 ± 0.1	2.1 ± 0.3			1.0 ± 0.1	1.4 ± 0.2
	ΔG	17.6	18.9			17.1	17.9
T	δ_0		7.62		1.74	6.16	
	CIS				−0.12	−0.14	
	K				30	20	25
	ΔG				8.4	7.4	7.9
TMP^{2-}	δ_0		7.62		1.74	6.16	
	CIS		−0.050		−0.070		
	$K \times 10^{-3}$		1.1 ± 0.2		0.70 ± 0.06		0.9 ± 0.2
	ΔG		17.3		16.2		16.8

[a] See footnotes to Table 2.

Table 7 Aminocalixarene affinity to double-stranded DNA, RNA and DNA model polymers; C_{50} (μM) values with ethidium bromide

	Charge in solution (pH = 7.0)	Poly(dA-dT)	CT-DNA	Poly(dG-dC)	Poly[A]-poly[U]
AC4a	+4	4.8	7.3	5.7	54.1
AC4c	+4	0.56	0.53	1.3	21.0
AC6	+6	0.28	0.32	0.41	37.4
AC8	+8	0.48	0.49	0.60	4.2
Spermine	+4	1.0	0.80	1.0	0.40

Concentration of ethidium bromide 1.26 μM; final concentration of DNA 3.6 μM.

moieties in the ligands can lead to substantial variations of affinity and selectivity towards nucleic acids,[12] the latter usually being low with simple aliphatic polyamines.[23a] It was of particular interest to see whether the larger aminocalixarenes can also generate conformational changes of double-stranded nucleic acids as found with some other macrocyclic polyamines.[25]

In the present first exploration we evaluated interactions between double-stranded (ds) nucleic acids and the four calixarene derivatives, of which **AC4c** and **AC4a** bear four positive charges, however, in quite different and relatively rigid orientations, whereas **AC6** and **AC8** have 6 or 8 charges and are relatively flexible. The calixarenes were again used as permethylammonium derivatives, with the advantage of pH-independent associations. It has been shown earlier[23b] that affinities with permethylated polyammonium compounds towards ds-DNA differ little from their protonated analogs, in line with the pre-

Table 8 Effect of aminocalixarenes on CT-DNA melting values (ΔT_m)

	Ratio[a]	ΔT_m/°C
AC4a[b]	0.05	+1.9
	0.1	+11.5
	0.2	+19.8
AC4c	0.05	+7.0
	0.1	+20.0
	0.2	+22.5
AC6[c]	0.05	+5.2
	0.1	+8.5
AC8[b]	0.05	+0.2
	0.1	+2.2
	0.2	+18.5
Spermine	0.1	+14.0

[a] Ratio of aminocalixarene to nucleobase. [b] Precipitation with higher ratio (0.3). [c] Precipitation with higher ratio (0.2).

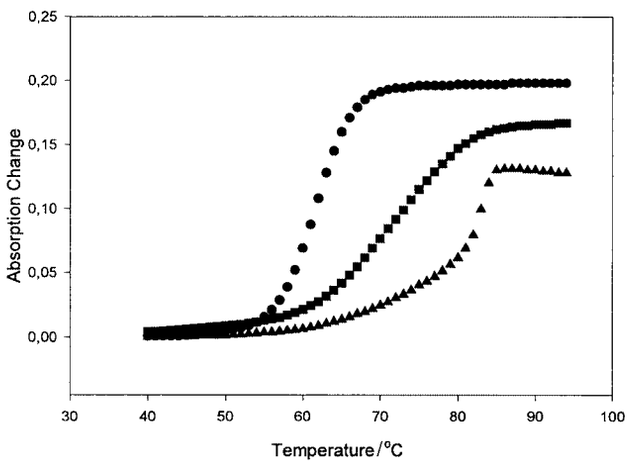


Fig. 3 A typical thermal denaturation curves in SHE buffer for CT-DNA (●), and its complexes with spermine (■) and **AC4c** (▲) at a ratio of 0.1 ligand per nucleobase/phosphate.

dominating electrostatic interaction mechanism. Affinities to ds nucleic acids were explored with thermal melting studies, which characterize the interaction by an increase of ΔT_m of the melting temperature (Fig. 3, Table 11), and by the fluorometric ethidium bromide assay (Table 8) also used before for polyamine–DNA interactions.[23] Here the concentration of ligand necessary for a reduction of the original fluorescence of the intercalated ethidium bromide EB by 50%, the so-called C_{50} value, is taken as a measure of the ligand affinity. Comparison of ΔT_m and in particular of the C_{50} values between the ligands **AC4c** and **AC4a** indicates a much larger affinity of **AC4c**, in spite of the same number of charges. Only in the cone conformer **AC4c** are the four charges located at one side and thus can interact simultaneously with the groove phosphates. The ΔT_m and the $1/C_{50}$ values of **AC4c** are higher than those observed with the equally charged aliphatic spermine (Tables 7,8), indicating also other binding contributions from the aromatic moieties. The larger calixarenes **AC6** and **AC8** show in spite of their larger number of positive charges only a very moderate affinity increase, also in comparison to spermine which has only four charges.

Preliminary molecular modeling with CHARMm, using an idealized B-DNA conformation as obtained from the QUANTA helix builder and energy minimized structures for the calixarenes, gives some insight into the geometric boundaries for contact ion pairs in the complexes. The distance between the phosphate in the B-DNA is 7 Å or 11 Å, respectively. The distance between opposite N^+atoms in the alternate conformer **AC4a** is 10 to 12 Å, which could allow formation of up to six salt bridges with the B-form of DNA. In the cone conformation **AC4c** the distance is 12 Å and 9 Å between opposite and neighbor N^+ atoms, respectively. Up to eight salt bridges with the B-form of DNA would be possible here. The compounds allow not only tight ion pairing with an essentially undistorted double-strand, but also some contacts of phenyl groups protruding into the groove. These interactions may explain the (relatively small) selectivities found between the different palindromic sequences studied (Table 7), with a general small preference for polydA*polydT in comparison to polydG*polydC. The most dramatic difference is seen with the RNA-mimic polyA*polyU, which with the calix[4] and calix[6] compounds shows much less affinity compared to DNA or DNA mimics (Table 7); this is in sharp contrast to spermine and also **AC8**, where there is much less discrimination. In **AC6** the distance between the N^+ groups in the A/D, in the A/C, and in the A/B aryl ring positions is 15 Å, 10–14 Å, and 8 Å, respectively. The simulations show only eight possible salt bridges to the DNA here. In **AC8** these distances are 18 to 20 Å (A/D), 12 to 18 Å (A/C); and 7 to 9 Å (A/B), respectively, with again eight possible salt bridges.

Solubility problems limited any detailed NMR analyses. The ^{1}H NMR spectra of aminocalixarenes such as **AC4c** shows (in 2×10^{-4} M concentration) with CT-DNA in concentrations of up to 1×10^{-3} M no appreciable line broadening (<5 Hz), which is in contrast to even weak intercalators where one finds consistently broadening of at least 25 Hz.[26] This excludes that a phenyl group intercalates into the DNA; in line with this there were no significant shift changes.

Conclusion

The presence of up to eight permethylammonium groups in the corresponding calixarenes allows us to bind nucleotides in water with constants up to 7×10^4 M^{-1}. With the calix[4] derivative a similar or even slightly increased affinity is observed with the 1,3-alternate conformation **AC4a** in comparison to the cone compound **AC4c**, although the latter has all four ammonium groups converging at one site. These, however, cannot be in simultaneous contact to the guest phosphate. The larger macrocycles **AC6** and **AC8** show only a moderate increase in affinity, and also an only moderately increased nucleobase discrimination. These observations agree with structures obtained by preliminary computer aided molecular modeling, showing that in all complexes the nucleobase and most of the sugar part are only partially enclosed in the calixarene cavity, but more so with the larger macrocycles. In line with this the observed NMR CIS values on the H-2 of AMP increase from 0.05 to 0.10 ppm, H-1′ from 0.05 to 0.09 ppm; the H-1′ shift of ADP increases from 0.09 to 0.14 ppm.

The interactions with DNA are again dominated by ion pairing and stabilize the double-strands, in contrast to some other macrocyclic polyamines where one found destabilization or base flipping. The observed small affinity differences and in particular the large NMR shielding effects seen with a nucleoside in spite of its much weaker binding indicate additional van der Waals-type binding contributions. The strong preference of the three calixarene derivatives **AC4c**, **AC4a** and **AC6** for DNA in comparison to RNA contrasts sharply with simple ligands such as spermine and may hold promise for biomedical applications.

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