Intercalation mechanisms with ds-DNA: binding modes and energy contributions with benzene, naphthalene, quinoline and indole derivatives including some antimalarials †

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Intercalation mechanisms into double-stranded calf thymus DNA have been probed with 38 different ligands, largely based on naphthalene or quinoline systems. It is shown how NMR shift and line width changes of the ligand signals can be used to unequivocally differentiate intercalation from groove binding modes, and to obtain by curve fitting association constants *K*. These show acceptable agreement if derived from several independent NMR signals. Less reliable information is obtained from selected UV titrations, from DNA melting differences, from calorimetric measurements and from affinity comparison to polyamines, the latter being based on a fluorescence assay with ethidium bromide.

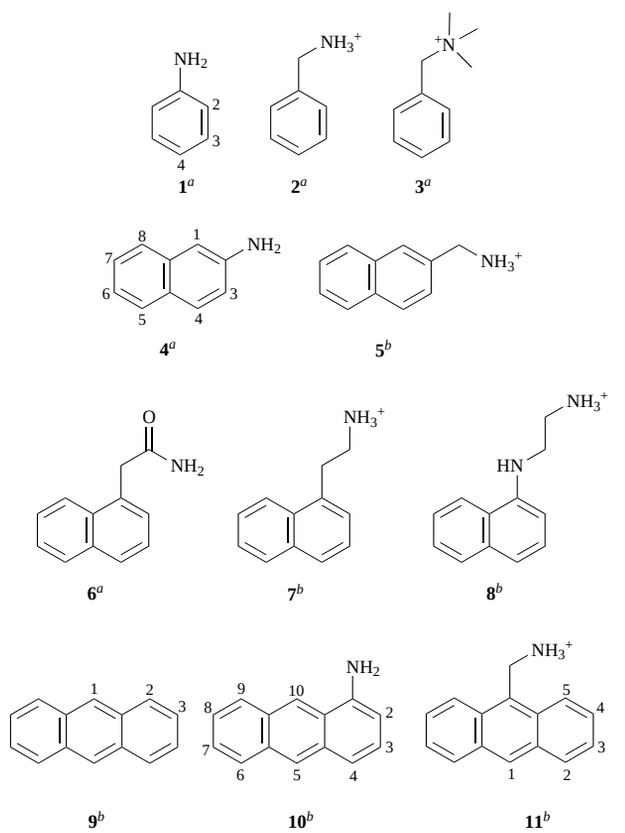
In contrast to literature expectations the naphthalene-shaped ligands show similar affinities irrespective of the presence of nitrogen atoms, or even of charges within the aromatic system. Quinolinium and naphthalene derivatives only intercalate if they bear a positively charged side chain, and then with similar binding constants. Comparison of all systems as well as salt effects demonstrate that the binding can be quantified with additive contributions from salt bridges of the ammonium centres in the side chains, and from the stacking effects of the aromatic parts. There is no evidence for non-classical intercalation by partial insertion between the nucleobases, nor for any intercalation of phenyl units.

Introduction

The understanding and quantification of DNA–ligand interactions [1,2] are of paramount importance for the study of biological mechanisms and for many applications such as drug design. Intercalation by stacking of smaller aromatic ligand moieties between the nucleobases plays a significant role in a multitude of DNA interactions.[3] There have been however; relatively few consistent experimental studies on the parent association energies and their dependence on ligand structure until now. One of the reasons for this is the frequent problems with the unambiguous characterization of the binding mode,[4] and of determining association constants.[5] One incentive for the present investigation was that the shielding effect of the aromatic nucleobases on NMR signals of stacked substrates [6] can provide direct insight into the intercalation mode. We have recently shown how both the change of NMR shifts and of signal width can be used to unequivocally distinguish intercalation from other binding modes, and to obtain reliable association constants from several independently followed signals.[7]

As a mechanistic challenge we try to answer experimentally the question whether unequal charge distribution, in particular positive charges within the ligand π-system, will enhance binding as suggested previously,[8] and whether weaker stacking ligands such as quinoline derivatives indeed show so-called non-classical intercalation by only partial insertion [9] between nucleobases, a concept which has been questioned already.[10] We furthermore wanted to explore the role of ammonium centres in the side chains of intercalators, and to see whether the total binding constants and their dependence on salt concentrations can become predictable on the basis of additive increments [11] of free binding energies.

The ligands chosen were largely naphthalene-shaped derivatives with positive charges placed at different positions within or outside the aromatic moiety. Smaller stacking units have the advantage of reflecting the consequences of distortions in the ligand structures better than large systems like acridines, which exhibit strong binding independent of structural details. Moreover, the intercalation process of anthracene-shaped ligands involves not only an additional set of base pairs on the other side of the helix [12] and hence possible steric distortions, but also



Structures of the carbocyclic compounds studied

[a] $\Delta\delta \leq 0.05$ ppm, $W_{\frac{1}{2}} < 5$ Hz, not intercalating. [b] $\Delta\delta$ or CIS: see Tables 1–3, usually $W_{\frac{1}{2}} > 10$ Hz (unresolved peaks), intercalating.

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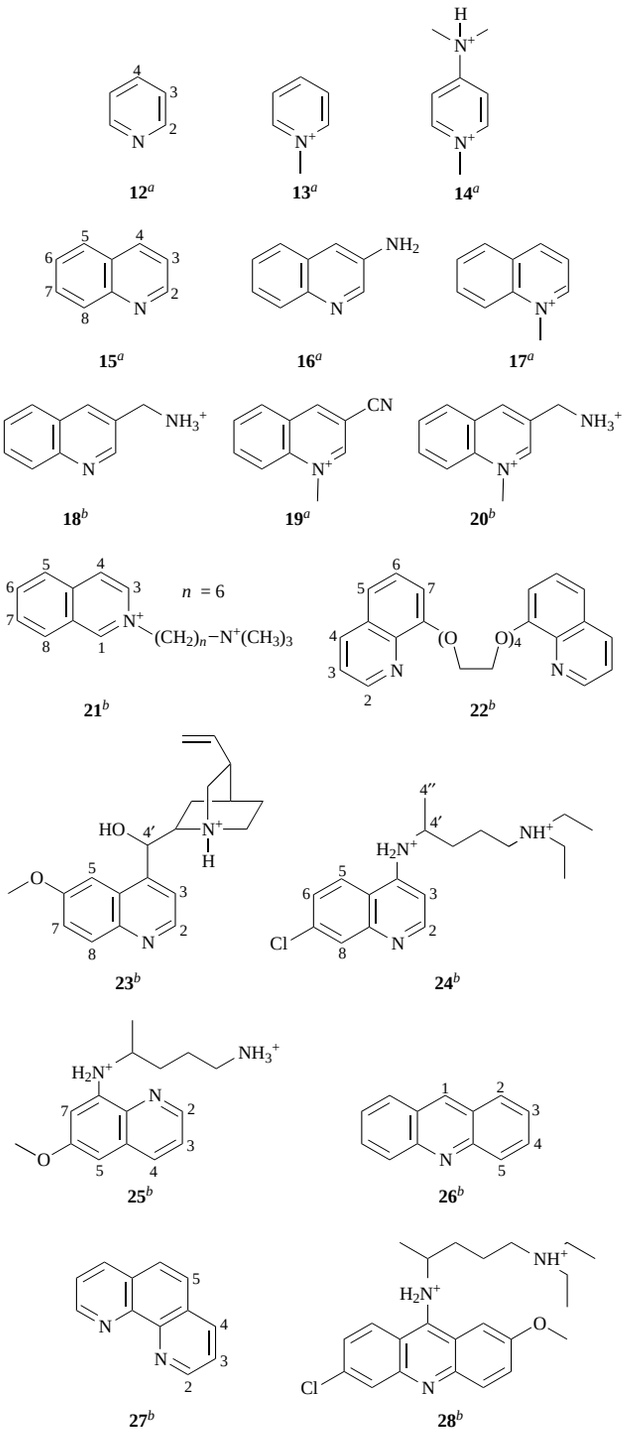

Structures of the heterocyclic compounds studied

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may interfere with the Watson–Crick hydrogen bonds.[13] Interactions between the positively charged protons of Watson–Crick hydrogen bonds and the central π-moiety of three-ring intercalators might furthermore contribute to their binding. Naphthalene-shaped derivatives are also of interest as they are the basis of several antimalarial and anticancer drugs. Clarification of the still disputed binding modes of these compounds[14] is another aim of the present work. Surprisingly, it seems to have remained unknown until now under which conditions quinine itself, or simple derivatives thereof, intercalate.

The importance of at least partial charges within the intercalating rings, or of multipolar fragments, has been stressed by recent *ab initio* molecular orbital calculations, describing stack-

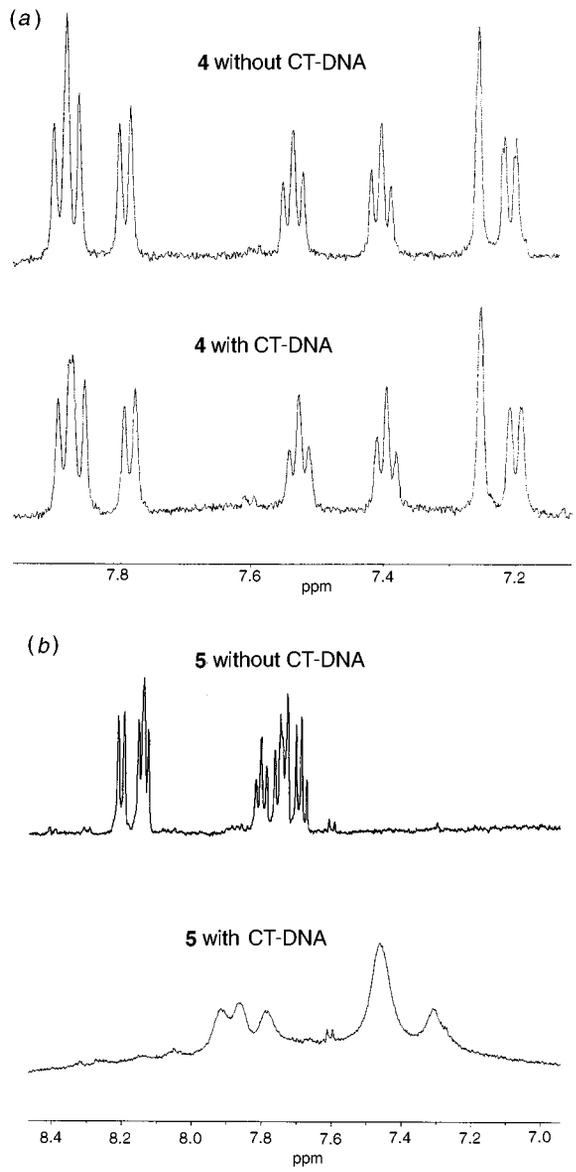


Fig. 1 ^{1}H NMR spectra of compound (*a*) **4** and (*b*) **5** with and without sonicated CT-DNA (D_2O, 20 mM Na phosphate, pD 7.4, $T = 303 \pm 0.1$ K, for concentrations of **4**, **5** and [DNA]; see Table 1)

ing mostly by electron correlation.[15,16] Corresponding charge distributions are also believed to be essential for a more qualitative π-charge sandwich model of nucleobase stacking.[16e] Exocyclic polar groups were thought to contribute significantly to stacking with the aromatic moieties by induction effects, which—in contrast to dominating dispersion interactions—would make extensive overlap of the π-surfaces less important.[17] This view is not in line with recent *ab initio* calculations favouring maximum overlap of the surfaces.[15a,d] Prediction of intercalation strength is hampered by rather extreme changes of partially compensating enthalpy and entropy contributions.[18] The almost prohibitive problem in accounting for entropy contributions in DNA complexes by free-energy perturbation calculations has been stressed recently in an investigation where the model was reduced to complexes of dinucleotides, although with large intercalators like doxorubicin.[19] The few available experimental investigations bearing on the intercalation strength are restricted either to comparison with biological activities,[19] or to model studies which for naphthalene predict stronger stacking with, *e.g.* adenine than with naphthalene itself.[20] It is hoped that new experimental data with a sufficiently large number of ligands will help to answer the partially conflicting conclusions on intercalations mechanisms. A sufficiently

Table 1 Results of the ^{1}H NMR spectroscopic titrations with sonicated calf thymus DNA and carbocyclic compounds (20 mM Na phosphate, pD 7.4, TSP as internal reference, $T = 303 \pm 0.1$ K)

Compound	Proton	$\Delta\delta$ [a]	CIS [b]	$\Delta G(\sigma)$ [c]/kJ mol^{-1}
1, **2**, **3** [d]	2-4, CH_2, CH_3	>-0.03		
4 [e,f]	1–8	$\geqslant-0.05$		
5 [e,g]	5	−0.28	−0.45	−19.2 (18.1)
	8	−0.28	−0.45	−18.8 (18.3)
	4	−0.35	−0.53	−19.6 (18.7)
	1	−0.38	−0.65	−18.8 (17.6)
	3, 6, 7	−0.28 to 0.35	—[h]	—
	CH_2	—[i]	—	—
6 [e,j]	2–8	$\geqslant-0.05$		
7 [e,k]	1	−0.41	−1.83	−13.9 (5.7)
	2	−0.40	—[l]	
	3	−0.41	−0.95	−16.9 (13.1)
	4	−0.50		
	5	−0.51		
8 [m]	1	−0.58	−1.25	−16.9 (10.5)
	2	−0.46	−0.65	−20.8 (—)
	3	−0.63	−1.56	−16.2 (11.1)
	4	−0.26	−0.70	−15.8 (10.8)
	5	−0.16	—[l]	
9 [n,o]	1–3	<-0.1		
10 [e,p]	2–10	−0.10 to −0.20		
11 [e,q]	1	−0.1		
	3, 4	$\leqslant-0.3$		
	2, 5	−0.1		

[a] Maximum of measured shift change, ppm. [b] Complexation induced shift (by nonlinear fit), ppm. [c] Standard deviation in parentheses. [d] See ref. 41; $[\mathbf{2}]_0 = 5.0$ mM, $[\mathbf{2}]_{end} = 2.16$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 10$ mM. [e] 10 vol% [$^2H_6$]DMSO. [f] $[\mathbf{4}]_{end} = 0.5$ mM, $[bp]_{end} = 7.5$ mM. [g] $[\mathbf{5}]_0 = 1.022$ mM, $[\mathbf{5}]_{end} = 0.75$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 2.5$ mM; $[\mathbf{5}]_0 = 1.6$ mM, $[\mathbf{5}]_{end} = 0.8$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 4.7$ mM. [h] No discrimination between overlapping signals possible. [i] Under HDO signal. [j] $[\mathbf{6}]_0 = 1.1$ mM, $[\mathbf{6}]_{end} = 0.67$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 2.2$ mM. [k] $[\mathbf{7}]_0 = 1.25$ mM, $[\mathbf{7}]_{end} = 0.9$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 2.5$ mM. [l] Not convergent. [m] $[\mathbf{8}]_0 = 9.41 \times 10^{-4}$ M, $[\mathbf{8}]_{end} = 6.72 \times 10^{-4}$ M, $[bp]_0 = 0$ M, $[bp]_{end} = 2.5$ mM. [n] 30 vol% [$^2H_6$]DMSO. [o] $[\mathbf{9}]_0 = 0.6$ mM, $[\mathbf{9}]_{end} \leqslant 0.3$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 2.6$ mM. [p] $[\mathbf{10}]_0 = 0.5$ mM; $[\mathbf{10}]_{end} = 0.45$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 1$ mM. [q] $[\mathbf{11}]_0 = 0.77$ mM, $[\mathbf{11}]_{end} = 0.43$ mM, $[bp]_0 = 0$ M, $[bp]_{end} = 1$ mM {concentrations at beginning ($[\ldots]_0$) and at the end ($[\ldots]_{end}$) of titration; M = mol dm^{-3}}.

large data set should also provide increments for free energy of binding, which can then be used for ligand design, and also can help us to understand DNA binding with natural systems including peptides and proteins.

Methods

NMR titrations

Fig. 1 illustrates the drastic differences in the NMR spectra of intercalating and non-intercalating naphthalene derivatives with calf thymus (CT-) DNA. The ligand **5** shift changes with increasing DNA concentrations were evaluated on the basis of the nearest neighbour exclusion principle (NNEP),[21] which assumes that intercalation will not occur from two sides of the same nucleobase. The non-linear least-square fits (Fig. 2) yield apparent binding constants K, which show gratifying agreement from four independent signals, with corresponding free binding energies ΔG differing by only 5% (Table 1). Scatchard plots with related tryptophan-containing peptides and CT-DNA[7] showed strong curvature, indicating—in contrast to literature reports[9]—strongly negative cooperativity as a consequence of the NNEP and/or ligand cooperativity. Although the Scatchard plot did not allow us to determine unambiguously the exclusion factor n and the cooperativity factor w independent of K, corresponding simulations showed $n \leqslant 4$ as the most likely value, which is in accord with the NNEP.[21] The NNEP requires that the total base pair concentration [bp] has to be multiplied by a factor of 0.5 for the fit. Application of other factors (for example 1.0 without NNEP) lowers the calculated ΔG values by about 5–10% on the average, the calculated complexation induced NMR shifts (see below) change even by usually <0.05 ppm (Tables 1–3).

Self-association can severely blur the observed NMR shifts, with large aromatic systems in particular, in which stronger aggregation leads to large shieldings comparable to, or even larger than those induced by intercalation between the nucleobases.[6b] The fit of a dilution experiment with chloroquine **24** showed that with substrates of this size the self-association constant with K *ca.* 3 is negligible. Furthermore, self-association will necessarily diminish the observed intercalation shifts, and would not be in line with the concurrent line width broadening which we see with all intercalating ligands (see below and ref. 7). DNA-promoted self association of ligands would also not be in line with the calculational model for fitting the titration curves, which shows no systematic deviations. In addition, the same fit, and within experimental error the same constant K was observed in an independent titration with a synthetic double stranded oligomer (to be published).

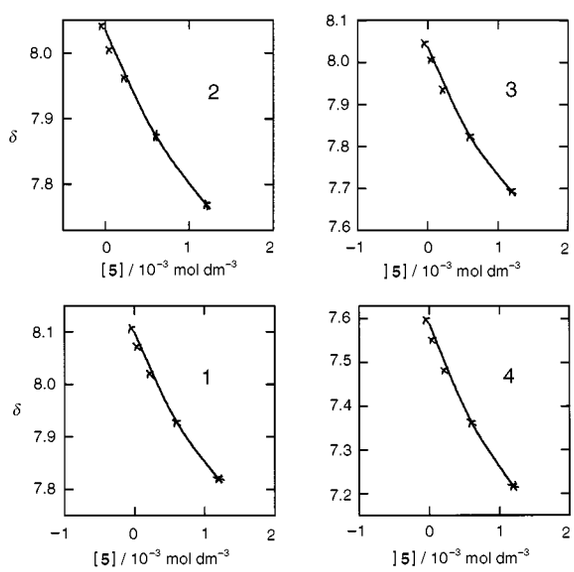


Fig. 2 NMR Titration with compound **5**; nonlinear fitting on the basis of next neighbour exclusion (D_2O, 20 mM Na phosphate, pD 7.4, $T = 303 \pm 0.1$ K, $[\mathbf{5}]_{end} = 0.75$ mM, $[bp]_{end} = 2.5$ mM); results see Table 1

Table 2 ^{1}H NMR spectroscopic measurements with heterocyclic compounds (conditions see Table 1)

Compound	Proton	$\Delta\delta$ [a]	CIS [b]	ΔG [c]/kJ mol^{-1}
12, **13**, **14** [d]	2–4, CH_3	$\geqslant -0.05$	—	—
15, **16**, **17** [e,f]	2–8, CH_3	$\geqslant -0.04$	—	—
18, **20** [g]	2–8, CH_2	$\leqslant -0.1$	—	—
19 [h]	2–8, CH_3	$\geqslant -0.05$	—	—
21 [i]	1	−0.16	—	—
	3	−0.25	—	—
	4	−0.25	—	—
	5	−0.22	—	—
	6	−0.17	—	—
	7	−0.21	—	—
	8	−0.12	—	—
	α-CH_2	$\leqslant -0.1$	—	—
	β-CH_2	−0.06	—	—
	γ/δ-CH_2	−0.01	—	—
	ε/τ-CH_2	±0.0	—	—
	CH_3	−0.01	—	—
22 [e,j]	2–7	$\leqslant -0.1$	—	—
23 [e,k]	2	−0.27	−0.34	−21.8
	3	−0.15	—	—
	5	−0.47	—	—
	6-OCH_3	−0.18	−0.21	−23.8
	7	−0.31	—	—
	8	−0.22	—	—
	4′	−0.12	−0.15	−23.3
23	Qui.[l]	±0.0		
	Olefinic	−0.03/+0.004		
24 [m]	2	−0.26	—	—
	3	−0.22	—	—
	5	−0.19	—	—
	6	−0.27	—	—
	8	−0.20	—	—
	4′	−0.13	—	—
	4″	−0.04	—	—
	CH_2	−0.02 to −0.04	—	—
	Ethyl	−0.002/+0.005	—	—
25 [e,n]	1	−0.52	−1.6	−14.8
	2	−0.43	−1.02	−16.0
	3	−0.44	−1.01	−16.3
	4	−0.50	−1.44	−15.2
26 [e,o]	1	−0.35	—	
	2, 5	−0.22	—	—
	3, 4	−0.4		
27 [e,p]	2	−0.29	—	—
	3	−0.39	—	
	4	−0.45	—	−19.8 [q]
	5	−0.40		
28 [r]	Aryl	$\geqslant -0.70$	—	—

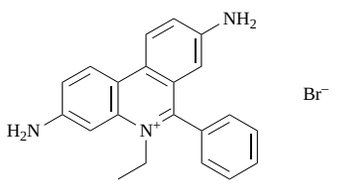
[a] Maximum of measured shift change, ppm. [b] Complexation induced shift (by nonlinear fit), ppm. [c] For next neighbour exclusion. [d] [Substrate]$_{end}$ = 0.5 mM, [bp]$_{end}$ = 10 mM. [e] 10 vol% [$^2H_6$]DMSO. [f] **[15]**$_{end}$ = 0.8 mM, [bp]$_{end}$ = 3.8 mM, **[16]**$_{end}$ = 0.9 mM, [bp]$_{end}$ = 2.1 mM, **[17]**$_{end}$ = 0.5 mM, [bp]$_{end}$ = 10 mM. [g] Not purified after synthesis, [substrate] (estimated) ≤1 mM, [bp] = 5 mM, shift change = mean value for complete group of overlapping aryl signals. [h] **[19]**$_{end}$ = 0.5 mM, [bp]$_{end}$ = 10 mM. [i] **[21]**$_{end}$ = 0.9 mM, [bp]$_{end}$ = 2.1 mM. [j] **[22]**$_{end}$ = 0.4 mM, [bp]$_{end}$ = 6.6 mM. [k] **[23]**$_{end}$ = 1 mM, [bp]$_{end}$ = 6 mM. [l] Mean value for all quinuclidene protons. [m] **[24]**$_{end}$ = 1 mM, [bp]$_{end}$ = 3.1 mM. [n] **[25]**$_{end}$ = 0.77 mM, [bp]$_{end}$ = 2.8 mM. [o] **[26]**$_{end}$ = 0.4 mM, [bp]$_{end}$ = 1 mM. [p] **[27]**$_{end}$ = 0.65 mM, [bp]$_{end}$ = 3.8 mM. [q] Estimated for 80% complexation. [r] See ref. 41.

In all cases where ligand signal separations were sufficient during NMR titrations [22] the CIS values as well as the half height width $W_{\frac{1}{2}}$ for 100% complexation could be obtained simultaneously with the K values from the curve fitting. In several cases CIS and $W_{\frac{1}{2}}$ values were obtained from single δ and W measurements at known concentrations and calculated with K constants known from other determinations. For other ligands only the actual shielding differences $\Delta\delta$ and/or $W_{\frac{1}{2}}$ values observed upon addition of DNA are given in the Tables. As shown previously with peptide–DNA complexes,[7] and again here, both CIS and $W_{\frac{1}{2}}$ values provide unequivocal differentiation between groove binding and intercalation.

Other methods for the analysis of affinity and binding mode

UV methods. The change of extinction coefficients of the intercalator by insertion into DNA has often been used to deduce association constants.[5,23] From the compounds investigated here only chloroquine **24** and acridine **26** have sufficient separation from the absorption maxima of the DNA. Even then, application of the method and line-fitting equations from the literature [24] yielded no fit for chloroquine, and only a poor fit for acridine; the value for acridine ($K = 4 \times 10^4$ M^{-1}) agrees roughly with the literature constant for proflavine [25] ($K = 10^5$ M^{-1}) (Table 4).

Affinity assay with the fluorescence dye ethidium bromide (EB). It has been shown that the fluorescence decrease by 50% for intercalated EB upon addition of ligands (the c_{50} value) is a



Ethidium bromide (EB) structure

linear function of the number of positive charges on the polyamine ligands,[26] and reflects their affinities even in the presence of permethylated nitrogen atoms.[27] The presence of additional binding fragments like an intercalating naphthalene unit in a ligand can lead to an additional binding, which therefore should be visible in a lowered c_{50} value. Indeed, ligands such as **21**–**25**, which by NMR are shown to intercalate, do exhibit c_{50} values which are lower by 10^2–10^3 than expected on the basis of their charge effect alone (Table 5). However, some ligands like the tripeptide **35** show a c_{50} value corresponding only to the number of charges, or even less although they do intercalate,[7] and methyl quinolinium iodide shows a c_{50} decrease by a factor of 10^2 although NMR clearly indicates the absence of intercalation. Low c_{50} values (or high affinities) in the absence of intercalation may here be the result of hydrophobic groove binding contributions. These have been shown to play a significant role for many groove-binding antibiotics.[28]

DNA melting effects. The traditional method for measuring ds-DNA stability changes [29] has been proposed to indicate also typical stability increases by intercalation.[8] The influence, for instance, of added salts on the melting point T_m can lead to misinterpretations,[30] with sometimes irregular behaviour due to DNA conformation changes.[31] Similar changes are reported [31] to occur upon addition of solvents like ethanol or dimethyl sulfoxide (DMSO). Solubility problems often forced us to use mixed solvents, which led to a linear decrease of T_m by up to 9 °C with 20 vol% EtOH (precipitation above this), and by up to 35 °C with 50 vol% DMSO.[32] All naphthalene- or indole-shaped ligands like **5** and **35** gave negligible T_m increases with CT–DNA (Table 6), even though they intercalate. Depending on concentrations, ionic strength and substrate one can even observe small T_m decreases by up to −3 °C (with **5** in the presence of 1 M NaCl), resulting from DNA conformation changes.

Calorimetry. According to literature reports [33] intercalation is characterized by a contribution of ΔH which is about twice as large as the $T\Delta S$ contribution to the total free binding energy ΔG. However, preliminary ΔH determinations, carried out as described elsewhere,[34] show such behaviour, and then only approximately, only with the strong intercalator EB and, to a lesser degree, with quinacrine **28** (Table 7). As solubility problems prohibit complete calorimetric titrations with DNA the ΔG values were obtained as far as possible from spectroscopic determinations. Small intercalators like **23**, **24**, **29** and **30** show $-5.4 < \Delta H < -0.4$ kJ mol^{-1}, and by comparison to ΔG sizeable

Table 3 ^{1}H NMR spectroscopic measurements with indole derivatives and indole and tyrosine containing peptides (conditions see Table 1)

	Gramine[d] **29**		Tryptamine[e] **30**		Trp[f] **31**	Lys-Trp[g] **34**
Protons	CIS[a]	ΔG^b/kJ mol^{-1}	CIS[a]	ΔG^b/kJ mol^{-1}	$\Delta\delta^c$	$\Delta\delta^c$
Trp 2	−0.67	−9.8	−0.40	−14.4	−0.01	−0.042
4	−0.70	−9.8	−0.30	−14.5	−0.013	−0.060
5	−0.63	−10.1	−0.29	−14.8	−0.012	−0.060
6	−1.30	−8.5	−0.40	−14.3	−0.013	−0.060
7	−0.73	−9.7	−0.32	−14.6	−0.013	−0.054
CH	—	—	—	—		—[l]
CH_2	—	—	—	—		−0.013
1-Lys CH	—	—	—	—		+0.08[a] −10.4[b]
CH_2 (1)	−0.08[c]	—	−0.07[c]	—		—
CH_2 (2)	—	—	−0.12[c]	—		—
CH_3	−0.03[c]	—	—	—		—

	Lys-Trp-Lys[h] **35**		Lys-Gly-Trp-Lys[i] **36**		Trp-Ala[j] **32**	Trp-Gly-Gly[k] **33**
Trp 2	−0.22	−17.6	−0.10	−16.4	−0.004	−0.01
4	−0.26	−17.6	−0.12	−15.9	−0.01	−0.008
5	−0.28	−17.5	−0.13	−16.4	−0.01	−0.004
6	−0.32	−17.1	−0.08	−16.4	−0.01	−0.009
7	−0.23	−17.7	−0.10	−16.4	−0.01	−0.01
CH	−0.12[c]	—	—			+0.03
						+0.05[a] −11.3[b]
CH_2	—[l]		—[l]			+0.011
1-Lys CH	+0.22	−18.0	+0.20	−16.5		
-CH_2	(0.00)[c]	—	(0.00)[c]	—		
					Ala:	Gly:
2-Lys CH	(0.002)[c]	—	(−0.001)[c]		CH −0.005	CH_2 +0.009/+0.03
-CH_2	(0.00)[c]	—	(0.00)[c]		CH_3 0.00	+0.009/+0.009

	37[m]	Lys-Tyr-Lys[n] **38**			
Protons	$\Delta\delta^c$	Protons	$\Delta\delta^c$	CIS[a]	ΔG^b/kJ mol^{-1}
1–4	⩽−0.1	ar1	−0.01		—
		ar2	−0.03		
		α-CH	+0.12	+0.28[o]	11.3[o]
		α-CH	+0.12	+0.21	16.5

[a] Complexation induced shift (by nonlinear fit), ppm. [b] For next neighbour exclusion. [c] Maximum of measured shift change, ppm. [d] **[29]**$_{end}$ = 0.42 mM, [bp]$_{end}$ = 9.6 mM, 10 vol% [$^2H_6$]DMSO. [e] **[30]**$_{end}$ = 0.17 mM, [bp]$_{end}$ = 10 mM. [f] **[31]**$_{end}$ = 0.4 mM, [bp]$_{end}$ = 7 mM, 10 vol% [$^2H_6$]DMSO. [g] **[34]**$_{end}$ = 0.23 mM, [bp]$_{end}$ = 7.5 mM. [h] **[35]**$_{end}$ = 0.47 mM, [bp]$_{end}$ = 4.0 mM. [i] **[36]**$_{end}$ = 0.21 mM, [bp]$_{end}$ = 5.1 mM. [j] **[32]**$_{end}$ = 0.6 mM, [bp]$_{end}$ = 3.3 mM. [k] **[33]**$_{end}$ = 0.46 mM, [bp]$_{end}$ = 7.1 mM. [l] Not determined because of overlapping and broadening. [m] **[37]**$_{end}$ = 0.3 mM, [bp]$_{end}$ = 2.8 mM, 10 vol% [$^2H_6$]DMSO. [n] **[38]**$_{end}$ = 0.55 mM, [bp]$_{end}$ = 4.4 mM. [o] Based on phosphate concentration.

Table 4 UV spectroscopic Scatchard analyses for compounds **24** and **26**[a]

Compound	$\Delta\lambda$/nm	ε_f[b]/dm^3 mol^{-1} cm^{-1}	ε_b[b]/dm^3 mol^{-1} cm^{-1}	K/M	σ[c]	n[d]	σ[c]	w[d]	σ[c]
24	3	18 800	7 097	—		—	—	—	—
26	3	12 269	8 385	5.3×10^4	$\pm 2.5 \times 10^3$	1.7	±1.6	0.8	±0.05
		12 269	8 384	4.1×10^4	$\pm 3.3 \times 10^4$	4.5	±2.0	0.5	±0.2
		12 269	8 384	3.3×10^4	$\pm 3.9 \times 10^3$	3.9	±3.6	0.5	±0.3

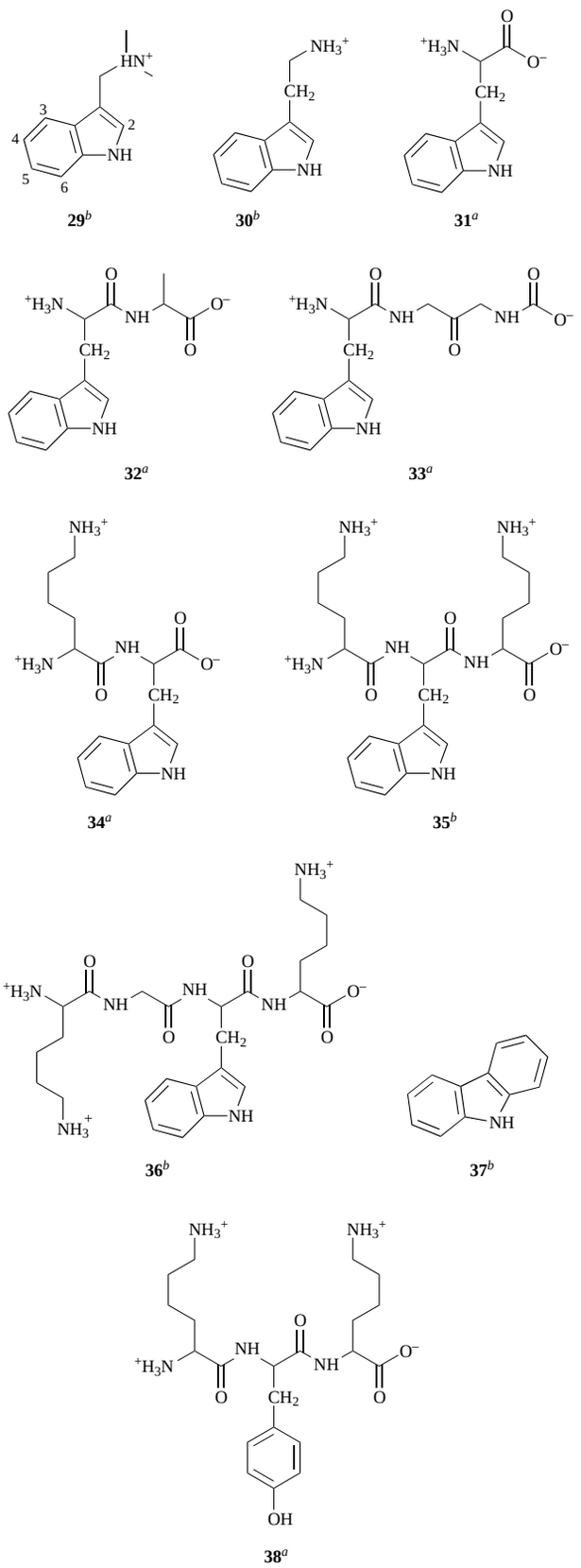
[a] 20 mM Na phosphate, pH 7.0, T = 298 ± 0.1 K, **[24]**$_{end}$ = 7.75×10^{-6} M; [DNA phosphate]$_{end}$ = 1.5 mM and **[26]**$_{end}$ = 1.6×10^{-5} M; [DNA phosphate]$_{end}$ = 8×10^{-4} M. [b] Molar extinction coefficients; ε_f: free substrate, ε_b: bound substrate. [c] Standard deviation. [d] n: exclusion factor; w: cooperativity factor.

positive entropic contributions (Table 7), likely due to desolvation effects. Surprisingly, such positive $T\Delta S$ terms are also observed with some strong intercalators (Table 7).[33b]

Results

Association of carbocyclic ligands 1–11

In line with observations on some-tryptophan containing peptides, and with earlier more qualitative results[6] intercalation of naphthalene parts is invariably characterized by strong upfield shifts (Table 1), which reach CIS values of up to −1.8 ppm as calculated from line fitting (as in Fig. 2). The actual magnitude depends on the position of the affected ligand protons with respect to the strongly asymmetric anisotropy cones of the nucleobases; it can thus vary significantly, but never drops below 0.15 ppm. Smaller changes occur only if an average of many protons are in rapid exchange, as is the case in highly symmetric compounds like the unsubstituted anthracene **9**. Another reason for smaller shift changes is that the protons of the large intercalators extending much over one purine or pyrimidine unit are partially placed near the edges of the shielding cones; by rapid exchange these protons can feel both shielding and deshielding effects of the nucleobase anisotropies. The line width $W_{\frac{1}{2}}$ has been shown to increase invariably with intercalating indole derivatives[7] to 50 Hz at 100% complexation. In the carbocyclic ligands the NMR signals are too close to allow the corresponding quantification. The line width $W_{\frac{1}{2}}$, however, for the naphthalenes with ammonium centres in the side chain is invariably above 10 Hz even at only a partial degree of complexation. In contrast, all other compounds show $W_{\frac{1}{2}} < 5$ Hz, and CIS (or, if unavailable, $\Delta\delta$) below 0.05 ppm.



Structures of the indole derivatives studied and indole or tyrosine containing peptides

[a] $\Delta\delta \leqslant 0.05$ ppm, $W_{\frac{1}{2}} < 5$ Hz, not intercalating. [b] $\Delta\delta$ or CIS: see Tables 1–3, usually $W_{\frac{1}{2}} > 10$ Hz (unresolved peaks), intercalating.

The NMR data indicate clearly for the first time that a phenyl ring, in contrast to earlier assumptions,[6a] will not intercalate in ds-DNA if it is not forced to do so by other binding contributions, and that naphthalene derivatives intercalate only if there are positive charges in a substituent side chain. Amide functions as in **6** are insufficient to enable naphthalene intercalation, although the affinity for DNA is sizable due to interactions in the grooves, reminiscent of groove-binding antibiotics like distamycin[2b] which also have lipophilic parts. The ΔG values for **5**, **7** and **8** average around 15 to 19 kJ mol^{-1}, depending slightly on the position of the side chain nitrogen centres; these energies can be described as the sum of stacking and electrostatic contributions (see below).

Heterocyclic aromatic ligands 12–38

The observed NMR shifts (Tables 2 and 3) and line widths lead to the same conclusion as with the carbocyclic ligands: quinoline, isoquinoline and indole rings show the same intercalation pattern if the rings bear positively charged side chains. The results with the methylquinolinium compound **17** show that even the presence of a permanent charge within the ring system does not lead to intercalation. That the *N*-methyl group does not hinder intercalation is obvious from the data for **20** which clearly intercalates. Again, the largest shift changes with up to −1.45 ppm CIS are seen with less symmetrical compounds like **24** and **25**. The three-ring systems **26** and **27** intercalate without the assistance from side chains, as discussed for the anthracene derivatives above. They all show small $\Delta\delta$ values again due to symmetry, but a characteristically enhanced line width. Within quinine **23** and primaquine **25** an NMR titration gave association constants of *ca.* 10^4 and 10^3 M^{-1} or, ΔG values of *ca.* −23 and −15 kJ mol^{-1} respectively. These values are slightly higher than those with the naphthalene derivatives, which is ascribed to the increased length and therefore flexibility of the side-chain residues.[33] The indole derivatives gramine **29** and tryptamine **30** clearly show intercalation of the aromatic moiety, as evident from CIS values[7] between −0.3 and −1.3 ppm as well as from $W_{\frac{1}{2}}$ values[7] of from 15 to 30 Hz. Nevertheless, the binding energies are, with $\Delta G = -9.7$, and −14.1 kJ mol^{-1} respectively, distinctly smaller than those of naphthalene-shaped derivatives. Similar results were obtained earlier with tryptophan-containing peptides,[7] which for intercalation even need support from two adjacent amino acids with positive charges.

Conclusions

Salt effects and additive binding contributions

The results demonstrate that aromatic systems comprised of two condensed rings can enforce destacking of the nucleobases and thus intercalation only if their association to ds-DNA is helped by positive charges in the side chains. Since placement of these charges obviously affects to some degree the total binding energies ΔG_t (compare values for *e.g.* **5** with **24**) we use the largest observed ΔG_t values measured with simple aminoalkyl-derivatives for the evaluation of the net contribution ΔG_{int} by intercalation. This is obtained by subtraction from ΔG_t a value of $\Delta G_{coul} = 5$ kJ mol^{-1} and per salt bridge, which we have found in about 70 ion paired complexes,[11] including DNA–polyamine interactions, usually assuming two salt bridges of one ammonium centre with two groove phosphates.[27] The comparison (Table 8) indicates that intercalation strength is essentially a function of the size of the aromatic moiety, independent of heteroelements within the π-system. Similar conclusions emerge from a systematic analysis of stacking forces between aromatic ligands and porphyrins in water.[37] Additive binding increments of single chromophores in depsipeptides containing quinoline chromophores have also been reported recently.[38] It should be stressed that the increments given in Table 8 represent—like others[38] measured with natural (calf thymus) DNA—only average values without nucleobase discrimination. Systematic replacement of nucleobases in well-defined homopolymer duplexes offers an attractive way to quantify the specific stacking mechanisms.[39] Corresponding recent results by Kool *et al.*[39] suggest that both

Table 5 Affinity measurements by ethidium displacement assay[a]

Compound	c_{50}/M	Compound	c_{50}/M	Compound	c_{50}/M
1	(>1.0)	**5**	—[b]		
2	0.216	**6**	—[b]	**9**	—[b]
3	0.43[c]	**7**	—[b]	**10**	—[b]
4	—[b]	**8**	—[b]	**11**	—[b]
12	(>0.4)[d]	**18**	—[e]	**24**	1.91×10^{-5}
13	>0.1[c]	**19**	—[b]	**25**	4.1×10^{-4}
14	>0.1[c]	**20**	—[e]	**26**	1.4×10^{-3}[g]
15	>0.01	**21**	7.5×10^{-4}	**27**	—[b]
16	—[b]	**22**	7.5×10^{-4}[f]	**28**	1.7×10^{-7}[c]
17	2.36×10^{-3}	**23**	6.17×10^{-4}		
		32	1.8×10^{-2}	**36**	5.2×10^{-3}
29	5.0×10^{-3}	**33**	2.9×10^{-2}[f]	**37**	—[b]
30	2.16×10^{-3}	**35**	(3.8×10^{-3})[h]	**38**	9.3×10^{-3}
31	—[b]	**34**	1.2×10^{-2}		

[a] 20 mM Na phosphate; pH 7.0; values corrected because of dilution and fluorescence of the substrates; $[bp]_0 = 1.7 \times 10^{-4}$ M; $[EB]_0 = 1.26 \times 10^{-6}$ M. [b] Not determinable because of limited solubility and precipitation. [c] See ref. 41. [d] Higher than the real c_{50} value because of limited solubility. [e] Not purified after synthesis. [f] 10 vol% DMSO. [g] 20 vol% DMSO. [h] Estimated value.

Table 6 ΔT_m values for selected compounds[a]

Compound	ΔT_m/°C	[Compound]/M	[bp]/M	Ratio [cpd]/[bp]
2	±0	1.3×10^{-3}	3.4×10^{-5}	30
5	+1.1	1.45×10^{-5}	7.1×10^{-5}	0.2
	+1.1	2.9×10^{-5}	7.1×10^{-5}	0.4
	+1.5	7.2×10^{-5}	7.3×10^{-5}	1
	+1.9	1.4×10^{-4}	7.1×10^{-5}	2
8	+1.1	1.7×10^{-4}	8.6×10^{-5}	2
15	±0	1.15×10^{-5}	2.3×10^{-6}	5
28	+0.1	5.96×10^{-5}	5.14×10^{-5}	1.2
	+3.6	1.96×10^{-4}	5×10^{-5}	3.9
34	−1.4	5.5×10^{-5}	5.33×10^{-5}	1
	−2.0	1.814×10^{-4}	5.04×10^{-5}	3.6
35	−0.1	3.6×10^{-5}	5.2×10^{-5}	0.7
	−1.8	1.77×10^{-4}	5.2×10^{-5}	3.4

[a] 20 mM Na phosphate, pH 7.0, $T = 298$ K.

Table 7 Microcalorimetrically determined ΔH values for selected compounds[a]

Compound	ΔH/kJ mol^{-1}	σ[b]	[Compound]/M	Ratio [bp]/[cpd]	ΔG/kJ mol^{-1}
EB	−38.2	±1.25	8.1×10^{-6}	31/1	−35.0[c]
23	−0.42	±0.13	1.1×10^{-5}	23/1	−21[d]
24	−3.55	±0.08	1.4×10^{-5}	18/1	−26.2[e]
28	−26.9	±1.1	7.3×10^{-6}	34/1	−37.0[f]
29	−1.25	±0.17	1.6×10^{-5}	15/1	−10 to −15[g]
30	−5.4	±0.4	2.1×10^{-5}	12/1	−7 to 10[g]

[a] 20 mM Na phosphate, pH 7.0, $T = 298 \pm 0.1$ K, $[bp]_{cell} = 0.25$ mM, cell volume = 1.4115 ml. [b] Standard deviation from three experiments. [c] See ref. 5. [d] NMR result: $K_{estimated} = 5000$ M^{-1}. [e] $K_{estimated} = 4 \times 10^{-4}$ M^{-1}. [f] See ref. 32. [g] From fitting of NMR titration.

size and polarity influence the stacking ability. However, the stacking in these experiments, where the variable unit is at the end of a duplex, as well as stacking in the experiments by Gellman *et al.*[20] may be influenced by solvophobic forces different from intercalation into the core of double stranded nucleic acids.

A consequence of the cooperativity between ion pairing and intercalation in these complexes is that the association constants must depend strongly on the ionic strength. This might be the major reason for literature discrepancies with, for instance, chloroquine **24**. With the NMR titration data from Table 1 we obtain association constants at different NaCl concentrations which now agree well with literature values. Another complication is the conformational change of B-DNA to Z-DNA or intermediate forms,[33] which are all less prepared for intercalation. For poly[GC]$_2$ these changes occur to 50% with NaCl at 4 M concentration; with spermine due to the many salt bridges they occur already at 4 mM.[33] Since the binding of the naphthalene-type ligands already is changing significantly at [NaCl] <1 M one can assume the B-DNA conformation to be stable under the measuring conditions, and can evaluate the salt effects on the basis of eqn. (1) given by Zimmermann *et al.*,[5,23b,d]

$$K = K1 + [K2/(1 + c_K K3)] \quad (1)$$

where K is the overall association constant, $K1$ constant for the intercalation process (not competing with ionic strength), $K2$ the one for the competing ion pairing, $K3$ the one of the competing salt, and c_K the salt concentration. The fit to eqn. (1) yielded negligible contributions for the non-competitive intercalation part (indicating $K1$ close to 0), but realistic values for the electrostatic contributions. With compound **5** one obtains with NaCl as salt (Fig. 3) a value of $K3 = 20$ M^{-1}; with ligand **23** $K3 = 50$ M^{-1}. Both constants are in satisfactory agreement with predicted constants for one to two salt bridges. This is also the case for the dependence on spermine for **5**, which yields $K3 = 2 \times 10^3$ M^{-1}, corresponding to about four salt bridges.

Experimental section

Materials

Most compounds were purchased from Aldrich and Fluka and used without further purification as their hydrochlorides or

Table 8 Overview on typical binding increments in water[a]

Residue	Example	No. of salt bridges n	ΔG_t/kJ mol^{-1}	ΔG_{int}/kJ mol^{-1}
Benzene[b]	**1**–**3**, **38**	1–2	<10	0
Indole	**29**	1–2	9.7	1–2
	30	2–3	14.3	1–2
	35	>3	17.3	1–2
	36	>3	16.2	≤1
Naphthalene	**5**	1–2	19–20	8–10
	7	1–2	15–16	8–10
	8	1–2	15–16	8–10
Quinoline	**23**	2–3	22–23	8–12
	24[c]	3–4	26.2	6–10
	25	1–2	15–16	6–10
Acridine	**26**	0	26–27	26–27[f]
	Proflavine[d]	0	25	25[f]
	28[e]	3–4	>30	10–15[g]
Salt bridge alone	Na^+, R_4N^+	1–2	5–10	0

[a] Total binding free energies ΔG_t taken from ligand with optimal binding; contribution from intercalation $\Delta G_{int} = \Delta G_t - \Delta G_{coul}$ with an average Coulomb energy of $\Delta G_{coul} = 10$ kJ mol^{-2} for two salt bridges, except as noted otherwise. [b] Extrapolated from parent c_{50} values. [c] See ref. 30. [d] See refs. 25(*b*) and 26(*b*). [e] See refs. 5 and 41. [f] Parallel insertion mode. [g] Different, head-on insertion mode of the acridine unit.

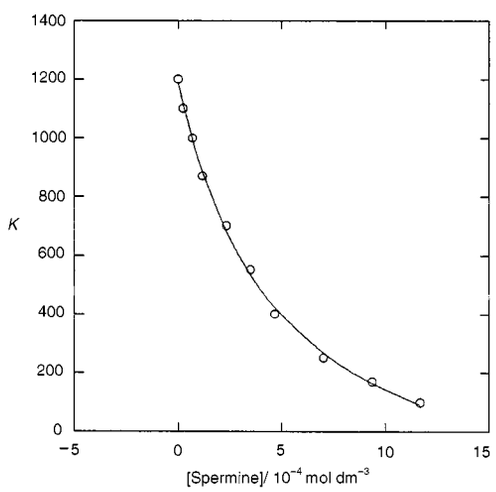


Fig. 3 Binding competition of compound **5** and spermine with sonicated CT-DNA. Estimated association constants K *vs.* spermine concentrations; nonlinear fitting to model described by Zimmermann,[5] eqn. (1), see text (D_2O, 20 mM Na phosphate, pD 7.4, $T = 303 \pm 0.1$ K).

phosphates, respectively (**23**–**25**, **28**). The peptides **31**–**36** and **38** were purchased from Bachem, and used as acetates in the case of the lysine ligands. Compound **3** was synthesized by permethylation with methyl iodide in dry DMF; a similar procedure was used in the case of the methylated pyridines or quinolines **13**, **14**, **17**, **19** and **20**. Compound **7** was available by reduction of compound **6** with borane in tetrahydrofuran (THF), and recrystallization from methanol. The hydrochloride **11** was synthesized *via* the Delepine reaction[38,39] from (chloromethyl)-anthracene. In reductions of the corresponding nitriles to compounds **18** and **20** with borane in THF only mixtures were obtained (>90% impurities). Compound **21** was synthesized by mixing isoquinoline and ω-bromohexyltrimethylammonium bromide (synthesis according to a literature procedure[40,41]) in dry DMF and heating to 80 °C for 1 h. The precipitate was recrystallized after cooling from isopropyl alcohol. All structures were characterized by NMR and as far as possible also by elemental analyses.[32]

Calf thymus (CT-) DNA was purchased from Aldrich as the sodium salt. Because of viscosity and solubility problems solutions of CT-DNA were sonicated according to literature procedures.[6b,42] The phosphate concentrations of the DNA solutions were determined by UV at $\lambda = 260$ nm ($\varepsilon = 6600$ dm^3 mol^{-1} cm^{-1}).

All measurements were taken in a 20 mM Na-phosphate buffer at pH 7.0 (Fluka). In order to increase the solubility of the substrates studied, different quantities of DMSO and [$^2H_6$]DMSO respectively were added (see Tables). Deuterated buffer was prepared by freeze-drying of the H_2O buffer and addition of D_2O (99.8%).

Equations for the nonlinear fitting and the Scatchard analyses were published earlier[24b,43] and applied using SIGMAPLOT 5.0 from Jandel Scientific. Microcalorimetric data were analysed with ORIGIN 3.16 from Microcal. Inc.

NMR studies

All spectra were recorded on a Bruker DRX 500 and processed with the programs UXNMR (Version 940401) and XWIN-NMR (Version 1.0) from Bruker. The temperature for all titrations with CT-DNA was 303 K (±0.1 K). The sodium salt of trimethylsilylpropionic acid (TSP) was used as internal reference.

UV studies

These were done with a Uvikon 860 from Kontron usually at 25 °C. For determining melting points at literature protocol[29] was followed, and the resulting first derivative of the absorption *vs.* temperature dependency was analysed by fitting to a Lorentzian curve. The temperature was controlled externally. Details of UV titrations and Scatchard analyses of DNA intercalation complexes were published earlier.[5,32] The extinction coefficient for the completely bound substrate (ε_b) was determined at 40-fold excess of DNA base pairs. The coefficient for the free substrate (ε_f) was the result of a dilution experiment in the concentration range where the Beer–Lambert law is valid.

Microcalorimetric studies

A microcalorimeter from Microcal. Inc. in the group of Blandamer was used.[34b] ΔH values were determined by single shot experiments at high excess of DNA base pairs and a temperature of 25 °C. Each experiment was prepared three times.

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