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Dimeric Calixarenes: A New Family of Major-Groove Binders

Wenbin Hu,[a] Caroline Blecking,[a] Marijeta Kralj,[b] Lidija Šuman,[b] Ivo Piantanida,[b] and Thomas Schrader*[a]

Abstract: A new class of potent DNA binding agents is presented. Dimeric calix[4]arenes with cationic groups at their upper rims and flexible alkyl bridges can be synthesized from triply acyl-protected calix[4]arene tetramines in relatively short synthetic sequences (3–5 steps). The compounds attach themselves to double-stranded nucleic acids in a noncovalent fashion, with micro- to nanomolar affinities. Guanidinium headgroups with their extended hydrogen-bonding "fingers" are more powerful than ammonium groups, and the benzylamine series is superior to the anilinium series (see below). The new ligands easily distinguish between RNA and various DNA types, and produce characteristic changes in UV/Vis, fluorescence, CD, as well as NMR spectra. Especially extended oligonucleotides of more than 100 base pairs are bound with affinities increasing from RNA (10 μM K_d) < AT-rich (1 μM) < GC-rich DNA double strands (100–10 nM). Ethidium bromide displacement studies confirm this order. CE_{50} values are remarkably low (1–4 μM), and are more than 300 times lower than that of spermine, which is a typical backbone binder. Stoichiometries are rather high (one calixarene dimer per two BP), suggesting a potential aggregation of bound ligands inside the major groove. Most UV/Vis melting curves display an inverted shape, and start from drastically enhanced absorption intensities for the DNA complexes. DAPI displacement studies prove that up to one equivalent of calixarene dimer can be accommodated in the dye-loaded DNA. RNA complexation by calixarene dimers is accompanied by a drastic CD spectral transition from the typical A-form to a perfect B-signature, providing further experimental evidence for major-groove binding. The orientation of the ligands can be deduced from NMR titrations and is reproduced in Monte-Carlo simulations on 1:1 complexes in water.

Keywords: calixarenes · circular dichroism · DNA recognition · fluorescent probes · hydrogen bonds · molecular recognition · UV/Vis spectroscopy

Introduction

Nucleic acid recognition is a fundamental biological process. It represents a major prerequisite for gene expression; all transcription factors and regulatory proteins dock onto a certain DNA segment and promote or inhibit transcription of the respective gene.[1] In addition, numerous other natural products are tailored for DNA recognition, as are many synthetic agents. These compounds all bind to different regions of the double stranded nucleic acid, with profound biological consequences. In fact, a high percentage of clinical antibiotics and antitumor agents are DNA binders.[2] Their mechanism of action mostly relies on intercalation (doxorubicin) and double strand disruption by lesions or cross links (for example, cisplatin and cyclophosphamide), which are often accompanied by serious unwanted side effects. Nature generally uses four different avenues for DNA recognition: intercalation into base pairs,[3] unspecific binding to the sugar phosphate backbone, and minor[4] as well as major groove insertion.[5] For the purpose of addressing a specific gene, the two latter cases are advantageous because, here, the base sequence becomes freely accessible and readable. Dervan et al. have developed a class of hairpin oligoamides that establish pairwise hydrogen-bond contacts to all base pair (bp) combinations and are thus capable of reading a 4–7 base fragment inside the minor groove. In cell-free assays[6] and isolated human erythrocytes, HIV-I transcription and, hence, viral replication could be effectively suppressed.[7]

Nature prefers to use the wider major groove, which has the right dimensions to accommodate cylinder-shaped peptidic α-helices. Protein motifs such as the zinc finger, leucine zipper, helix–turn–helix and helix–loop–helix structures are all well-suited for this purpose.[8] Molecular recognition of base pairs occurs by means of hydrogen bonds, preferential-

[a] Dr. W. Hu,[+] Dr. C. Blecking,[+] Prof. Dr. T. Schrader
Department of Chemistry, Universität Duisburg-Essen
Universitätsstr. 7, 45117 Essen (Germany)

[b] Dr. M. Kralj, Dr. L. Šuman, Dr. I. Piantanida
Divisions of Chemistry
Biochemistry and Molecular Medicine
Ruđer Bošković Institute, Bijenička 54
10000 Zagreb (Croatia)

[+] Both authors contributed equally to this work.

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ly with basic (Arg, Lys) and amidic (Asn, Gln) amino acids. Such residues also further stabilize the complex by forming salt bridges with backbone phosphate anions.[9]

Under certain conditions, oligonucleotides with purine bases can dock onto existing double helices at the Hogsteen sites and form triple helices, which prevent the approach of regulatory proteins.[10] A more general and simplified concept was introduced by Nielsen, who designed uncharged peptide nucleic acids (PNA) that generate a $(DNA)(PNA)_2$ triplex.[11]

Surprisingly, very few purely noncovalent major-groove binders are known. Mascarenas et al. combined a tripyrrole with a short leucine zipper or zinc finger motif for simultaneous minor/major groove fixation, and achieved sequence-selective binding to double-stranded DNA.[12] In 2001, the Hannon group synthesized a nanocylinder that snugly fits into the major groove of DNA.[13] This supramolecular Fe^{2+}-bispyridylimine cylinder induces DNA bending and coiling and stabilizes three-way junctions;[14] another largely inorganic construct is Schmid's Au_{55} gold cluster, which leads to necrosis of tumor cells and is also calculated to perfectly fit into the dimensions of the major groove of DNA.[15]

For more than a decade it was known that cationic calix[4]arenes display high affinities towards DNA. Schneider,[16] Ungaro,[17] and others synthesized trialkylammonium and guanidinium species and demonstrated complex formation by electrophoretic mobility assays. However, it was argued that the calixarenes would recognize the backbone phosphate anions, and multiple electrostatic interactions would ultimately lead to DNA coiling, which was finally proven by using AFM techniques.[18]

We recently developed dimeric anilino-calixarenes with simple alkyl spacers as new potent DNA-binding agents and studied the influence of extended spacers with or without additional positive charges.[19] The results indicated that affinities can be optimized and that large sections of the DNA (including full promotor sequences) can be simultaneously complexed by a single calixarene dimer.[20] This might pave the way towards sequence-selective DNA recognition, provided that major groove insertion can be proven to be the underlying binding mechanism and that the spacer can be replaced by recognition units for nucleic acid base pairs. This work accumulates pieces of evidence for the postulated major groove binding mode.

Results and Discussion

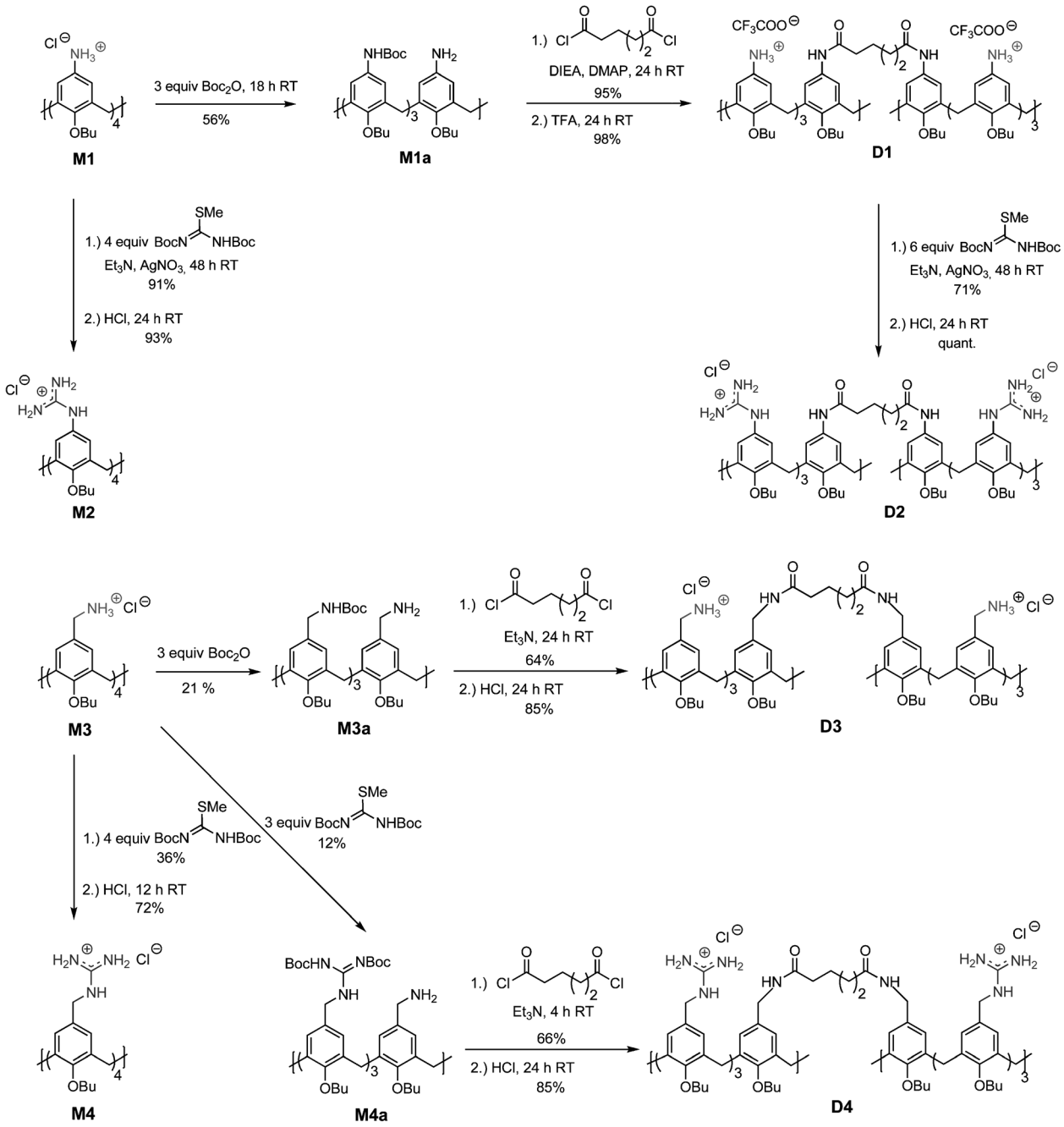
Without a crystal or NMR structure, it is not a trivial task to elucidate the exact mechanism of a new DNA-binding agent. Intercalation can be ruled out because of the dimensions of even the most compact anilinocalixarene in its preferred pinched cone conformation (upper rim diameter: 11 Å, lower rim: 8 Å).[21] However, backbone recognition and minor groove insertion must be taken into account as alternatives to the proposed major-groove binding mode. To broaden the scope of this investigation, we decided to synthesize four different classes of calixarene dimers that differ only in their cationic functionalization at the upper rim: anilinocalixarenes **1** and the related guanidinium species **2**, as well as the aminobenzyl calixarenes **3** and their guanidinium counterparts **4**. The extension to the benzyl series turned out to be a great leap forward with respect to potential applications; in contrast to the anilino prototype, the new dimers **3** and **4** are perfectly water-soluble in up to millimolar concentrations, and their DNA affinities are approximately 100 times higher than those of dimer **1**.

Nature uses guanidinium ions extensively to establish ion-pair-reinforced hydrogen bonds to nucleic bases and phosphate anions surrounding the major groove of DNA (see above).[22] Arginines are key players among those residues used for noncovalent base reading. For a better comparison, we also synthesized the respective monomeric calix[4]arene species, giving rise to eight test objects, depicted in Scheme 1.

The synthesis of all dimers commenced with the preparation of the monomeric symmetrical calix[4]arene amines **M1** and **M3**.[23] These were smoothly converted into the respective guanidine monomers **M2** and **M4** by successive treatment with *N,N'*-bis-Boc(2-methyl-2-thiopseudourea), followed by mild hydrolysis with HCl in dioxane. From **M1** and **M3**, a straightforward synthetic sequence led to the related dimers. Triple Boc-protection left only one amine moiety free, which was allowed to react with adipoyl dichloride on both ends to furnish, after Boc removal with trifluoroacetic acid (TFA), the dimeric calixarene amines **D1** and **D3**. Synthesis of the guanidine dimers was more complex: Compound **D2** was generated from **D1** after careful treatment with six equivalents of a guanidinylating agent and final mild hydrolysis. Compound **D4** was obtained from **M3** by triple guanidinylation, extensive chromatographic purification, coupling, and treatment with methanolic HCl. Monomers and dimers were stored as their respective trifluoroacetate or chloride salts.

The calixarenes substantially differ in their p*K* values: whereas both guanidinium dimers display p*K* values of up to 12, the benzylamine derivative reaches a p*K* value of 10,[24] but the aniline derivative only p*K* 5 (in the first protonation step). Thus, at physiological pH 7.0, compounds **D2**, **D3**, and **D4** are essentially hexaprotonated, whereas **D1** carries only two positive charges, which is a disadvantage if DNA complexation relied solely on electrostatic attraction. Another striking difference between the aniline and the benzylamine series is their water solubility; compounds **D1** and **D2** are only soluble in buffered 1:1 methanol/water mixtures, whereas millimolar solutions of **D3** and **D4** can be prepared in aqueous buffer at physiological salt loads.

Dimeric as well as monomeric calixarenes were subjected to a wide range of DNA and RNA binding experiments on nucleic acid substrates of varying length (12 to 10,000 BP). In addition to evaluation of cytotoxicity in tumor cell lines, these binding studies included fluorescence titrations, ethidium bromide (EB) and diamidinophenylindole (DAPI) displacement, UV/Vis melting curves, circular dichroism (CD)



Scheme 1. Synthetic pathways towards monomeric and dimeric calixarene derivatives **M1**–**D4** for the elucidation of their DNA-binding mechanism.

spectroscopy, gel permeation chromatography (GPC) measurements, NMR spectroscopy, and MD simulations. Taken together, all our results (see the Supporting Information) support our assumption that the calixarenes studied here are inserted into the major groove.

Fluorescence titrations: Direct titration of fluorescence-labeled nucleic acids with the new calixarenes leads to total quenching and revealed strong binding (i.e., micromolar to 10 nM K_d) of all derivatives (Table 1).[25] Compound **D1**, with only two instead of six charges, is an exception; this would indicate dominant electrostatic contributions that could be significantly attenuated by high salt loads. Impor-

Table 1. Affinities between selected calixarenes and oligonucleotides, expressed as K_d values (stoichiometric ratio dimer/base pair).

Dimer	12 GC	12 AT	12 AU
D1	1.7 μM (1.2:1)	2.5 μM (1.8:1)	10 μM (1.4:1)
Dimer	$(GC)_{10}$	$(AT)_{10}$	$A_{20}U_{20}$
D2	140 nM (0.6:1)	330 nM (0.6:1)	500 nM (0.6:1)
D3	1.0 μM (0.6:1)	1.7 μM (0.5:1)	5.0 μM (1.2:1)
D4	200 nM (0.6:1)	500 nM (0.7:1)	1.3 μM (0.8:1)

tantly, the benzyl series reached the same K_d values as the anilino series, however, at physiological conditions; direct comparison in the same solvent composition revealed a factor of 100 in favor of the benzyl calixarenes, the cations of which are electronically separated from the arene cores. Among each series, guanidinium derivatives often displayed superior affinities over the ammonium derivatives; this effect may well originate from the extended hydrogen-bond donor "fingers" of the guanidinium hand, which are able to reach not only the nucleic bases, but also span the entire major groove and touch the anionic phosphodiester backbone (see modeling section below). Another clear trend is markedly weaker RNA than DNA binding, which could indicate a better fit into the wider, major groove of DNA (see also ethidium bromide displacement).

In general, complex stoichiometries seem to be related to ligand charge.[26] Thus, the number of $\mathbf{D1}^{2+}$ dimers per base pair is approximately 1.8, whereas the ratio of $\mathbf{D2}^{6+}$, $\mathbf{D3}^{6+}$, or $\mathbf{D4}^{6+}$ to base pair is about 0.6:1 (for comparability and clarity, all nucleic acid concentrations are expressed in base pairs, and stoichiometries or equivalents of ligands are related to base pairs accordingly).

A linear alignment of dimer molecules inside the major groove would produce much lower stoichiometries (ca. 1:6), but would also leave much space unexplored. As a result, calixarene dimers with higher charges especially would be expected to form aggregates inside the major groove of DNA, presumably with favorable van der Waals contacts between their butoxy tails and unpolar arene faces; however, no oligomers were found for pure dimers by GPC analysis. Affinities for each calixarene unit also increased significantly with DNA length, pointing to a potential clamping effect on the loose strand ends. Monomers behave in a similar way to dimers, and thus also qualify as potential major-groove binders.

It is well-known that the minor groove of poly(GC) strands is sterically less accessible to bulky guests due to the presence of guanine's amino group, whereas the methyl substituents of thymine in poly(AT) protrude into the major groove.[27] A typical minor-groove binder therefore strongly prefers poly(AT); this is not the case with the dimeric calixarenes investigated here, thus ruling out minor-groove insertion (Figure 1).

To generate a preliminary binding profile, a wide range of small biomolecules, including peptides and sugars, was screened against dimeric calixarene **D1** and showed small affinities.

Ethidium bromide displacement assay: Ethidium bromide (EB) is the classical staining reagent in DNA electrophoresis; it intercalates into almost every second base pair and excitation at 510 nm then induces a strong fluorescence emission at 595 nm ($K_d \approx 100$ nM).[28] DNA intercalators and also strong groove-binders can compete with the dye and expel it from its complexation site, leading to reduced fluorescence emission.[29] The characteristic C_{50} value (ligand concentration for 50% fluorescence reduction) is indirectly proportional to the apparent binding constant of the DNA ligand. Smaller C_{50} values thus represent a superior DNA affinity. Complete dye displacement was achieved with all calixarene derivatives, with C_{50} values of around 10 μM,[30] but most notably very low CE_{50} values (charge excess of the EB-displacing ligand)[31] around 1–5 were calculated for the dimeric calixarenes, including **D1**; thus, clearly, more than Coulomb forces hold the ligands in place (Table 2). At physiological salt load, the dimer–DNA interaction only slightly weakened, which is another strong case for groove insertion as opposed to mere phosphate anion recognition. In contrast, backbone binders such as spermine and spermidine, which employ electrostatics almost exclusively, feature CE_{50} values 1000 times higher under these conditions (calf thymus DNA + **D1**: 1.7 μM; spermine: 1500 μM). We interpret this result as strong evidence for groove binding, for which additional hydrophobic forces and hydrogen bonds can be exploited.[32] Again, no profound difference could be found between the results obtained with monomers **M1–4** and dimers **D1–4**.

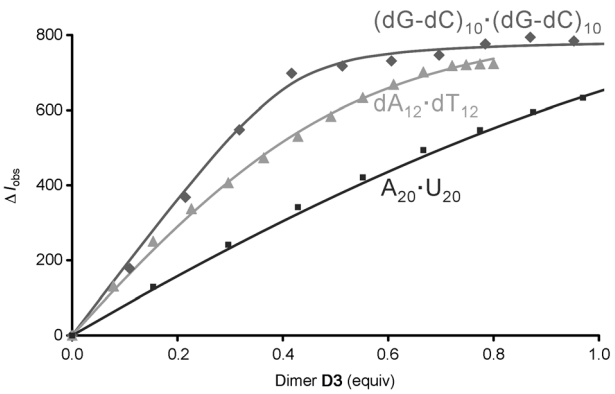


Figure 1. Representative fluorescence titration curves/binding isotherms for dimer **D3** and various nucleic acids of comparable length; distinguishing between DNA and RNA compositions/types.

Table 2. Corresponding CE_{50} values for complexes between **D3** and polynucleotides (>100 mers) of varying composition. [Nucleic acid] = 1 μM in base pairs; [EB] = 1.26 μM in base pairs.

GC-rich		AT-rich				AU-rich	
red	purple	dark-green	light-green	olive	khaki	dark-blue	light-blue
1.1	1.5	5.2	5.8	6.3	6.4	18.4	>30

Transition to very long oligonucleotides (12 /20 /100 mer) sharpens the distinction between nucleic acid strands of different conformation and steric hindrance: With these substrates, poly(GC) is much better bound by calixerene dimers, and poly(AT) and RNA much worse than random sequence DNA; these parallel results also suggest a superb fit into the wide major groove of DNA (Figure 2).

UV/Vis melting curves: Heating DNA solutions leads to a characteristic increase of UV absorbance due to dissociation of the double strand; the inflection point of the sigmoidal curve is called the melting temperature T_m (50% strand dis-

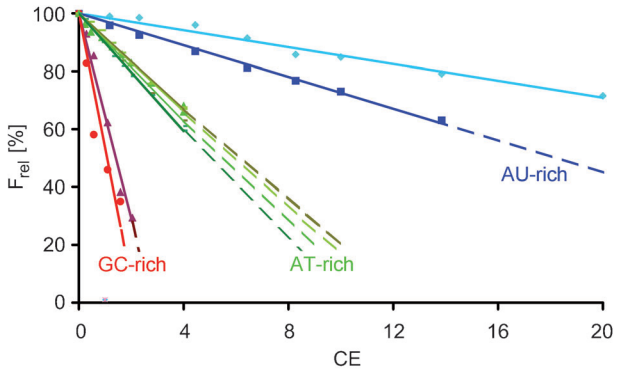


Figure 2. Representative ethidium bromide displacement curves for three major families of unlabeled nucleic acid duplexes (0.5 μM in base pairs) by dimer **D3** (20 mM phosphate, pH 7.0): GC-rich DNA > AT-rich DNA > RNA (all > 100 mers). (Red) $[(dGdC)_n]_2$+**D4**; (purple) $[(dGdC)_n]_2$+**D3**; (dark-green) $[(dAdT)_n]_2$+**D4**; (light-green) A_nT_n+**D3**; (olive) A_nT_n+**D4**; (brown) $[(dAdT)_n]_2$+**D3**; (dark-blue) A_nU_n+**D4**; (light-blue) A_nU_n+**D3.** F_{rel} = relative fluorescence emission intensity; CE = charge excess ratio.

sociation); this value indicates the thermal stability of the double helix.[33] Intercalators and groove binders usually stabilize DNA strands and increase the T_m values.[34] The normal profile of the melting curves for most examined DNA derivatives totally change upon complex formation with calixarene dimers, especially in methanol-containing aqueous buffer. In the presence of **D1–D4**, inverted melting curves appeared with drastically increased initial UV/Vis absorptions for the double-stranded DNA calixarene complexes; no temperature dependence was found for the ligands themselves (Figure 3). These changes generally suggest involvement of the DNA chromophores in the calixarene complex, for example, by way of direct hydrogen-bonding to the NH groups of the calixarenes. In extreme cases, inflection points increase by more than 40 °C, especially in complexes with guanidinium calixarenes, which can span the whole major groove (Table 3, see also titrations). Intriguingly, this effect, which most likely indicates DNA stabilization, is pronounced for short DNA strands (12 mers) and diminishes in complexes with longer oligonucleotides (20 mers); this is most likely another argument for strand-end chelation. This time the monomeric calixarenes fall short and hardly stabilize DNA strands at all. We tentatively explain this behavior with a two-point binding mode of the dimer, which ensures a connection between both phosphodiester backbones.

Table 3. T_m and ΔT_m values for complexes between selected calixarenes and oligonucleotides.

Calixarene[a]	20 bp DNA		20 bp RNA	
	T_m [°C]	ΔT_m [°C]	T_m [°C]	ΔT_m [°C]
D1	59	+16	64	+23
D2	>85	> +42	47	+06
M1	42	−01	57	+16
M2	51	+08	50	+09

[a] In 2 mM Hepes in MeOH/water (1:1), 9.4 mM NaCl, pH 7.1

Circular dichroism spectra: To gain a deeper insight into the changes of polynucleotide properties induced by calixarene binding, we chose to use CD spectroscopy as a highly sensitive method with which to detect conformational changes in the secondary structure of polynucleotides.[35] Moreover, achiral small molecules can eventually acquire an induced CD spectrum (ICD) upon binding to polynucleotides, which could give useful information about their modes of interaction.[36]

CD spectra of various DNA and RNA sequences with or without the calixarene ligands were measured at 10 μM concentrations in aqueous buffer. Although the calixarene monomers and dimers are achiral and, hence, CD silent, characteristic changes were observed in the CD spectra of their DNA/RNA complexes, with a much more sensitive distinction between long oligonucleotides (100 mers). Molar ellipticities decreased at all wavelengths and were proportional to ligand concentration, which is possibly a consequence of the nucleic acid helix unwinding with concomitant groove widening to accommodate the sterically demanding calixarene guest. Isoelliptic points demonstrate the existence of a single, well-defined, complex geometry. Monomeric calixarenes, with their spherical shape, produce much weaker effects. RNA complexes displayed the most significant changes. CD

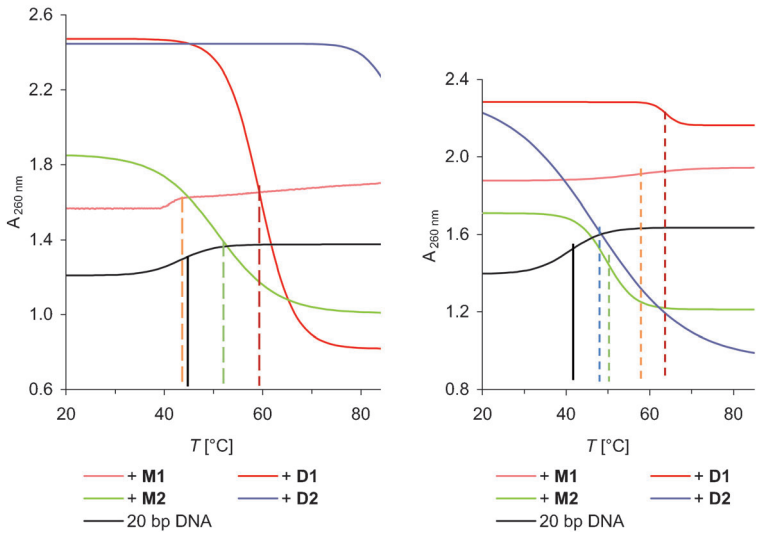


Figure 3. Representative UV/Vis melting curves for dimers and monomers **1** and **2** with 20 BP ds DNA (left) and 20 BP ds RNA (right, both in 2 mM Hepes, 9.4 mM NaCl, MeOH/H_2O 1:1, pH 7.1). Note the drastic DNA inflection point increase brought about by both dimers, which is absent in complexes with the respective monomers. RNA behaves differently.

maxima continuously shifted during an RNA titration with a calixarene dimer; finally, the new CD spectrum closely resembled that of B-DNA (Figure 4, Table 4). This conformational change further explains the lowered free-binding energy in terms of enthalpic cost. We interpret this result as compelling evidence for the postulated major-groove binding mechanism of all calixarene dimers.

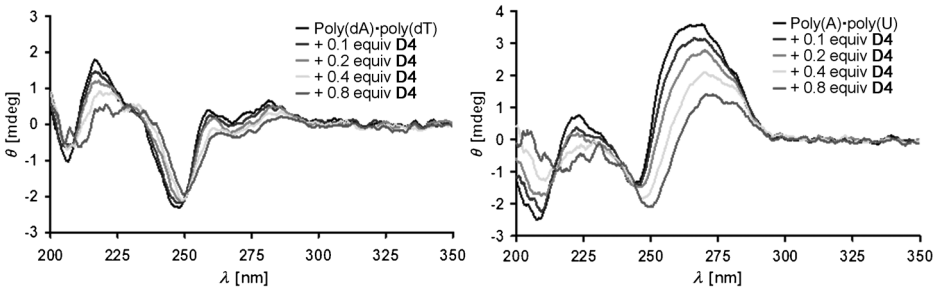
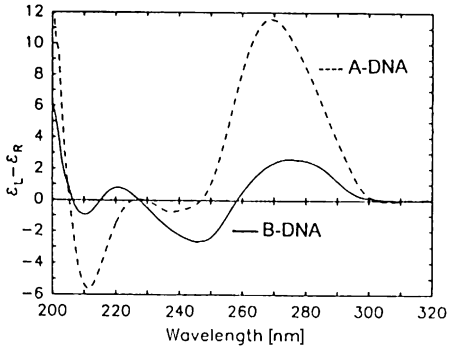


Figure 4. CD titration series for DNA (left) and RNA (right) complexed by dimer **D4**. Note the similarity between the final induced red RNA CD curve and naturally occurring B-DNA (see Table 4).

DAPI displacement assay: The dye diamidinophenylindole **(**DAPI) preferentially binds to poly(AT) DNA and leads to formation of a strong induced CD band at 376 nm (strong emission at 458 nm). Although DAPI binds to the minor groove of DNA, its affinity is only mediocre (10 μM) and DAPI is therefore generally used in displacement experiments with more powerful DNA binders.[38] Calixarene dimers **D1**–**D4**, with their submicromolar K_d values, appear well-suited for such investigations. Consequently, very low CE_{50} values (around 1 μM per BP) were determined in fluorimetric experiments that were comparable to those found with EB (see the Supporting Information). However, CD experiments revealed that, initially, stoichiometric amounts of the calixarene dimers are bound to DNA, before the induced CD band declines and DAPI displacement begins (Figure 5).[39] This experiment, which was reproduced many times, suggests that DAPI and stoichiometric amounts of calixarene dimers can be bound simultaneously by double-stranded poly(AT) DNA. Because DAPI, with its flat extended π-face, occupies the minor groove, the dimeric calixarene most likely resides in the major groove.[40] Monomers do not show this effect.

NMR spectroscopic experiments: Dimer **D1** was titrated with increasing amounts of a self-complementary DNA strand (Dickerson dodecamer 5′-CGC GAA TTC GCG-3′).[41] All aromatic as well as the diastereotopic methylene protons of the calixarene ligands experienced substantial chemical shift changes, whereas the butoxy tails remained unaffected (Figure 6).[42] Clearly, the calixarene heads penetrate deeply into major groove of the DNA and experience an altered local magnetic field. Hydrogen-bond contacts be-

Table 4. Groove widths and depths for selected nucleic acid conformations. Note the drastic changes accompanied with an A-to-B DNA helix transition.[37] The Figure was reproduced from ref. [36].



Structure type	Groove width [Å]		Groove depth [Å]	
	Major	Minor	Major	Minor
A-DNA	**2.7**	**11.0**	**13.5**	**2.8**
A_nU_n	3.8	10.9	–	–
B-DNA	**11.7**	**5.7**	**8.5**	**7.5**
$(dGdC)_n$	13.5	9.5	10.0	7.2
$(dAdT)_n$	11.2	6.3	–	–
C-DNA	**10.5**	**4.8**	**7.5**	**7.9**
$d(A)_nd(T)_n$	11.4	3.3	–	–

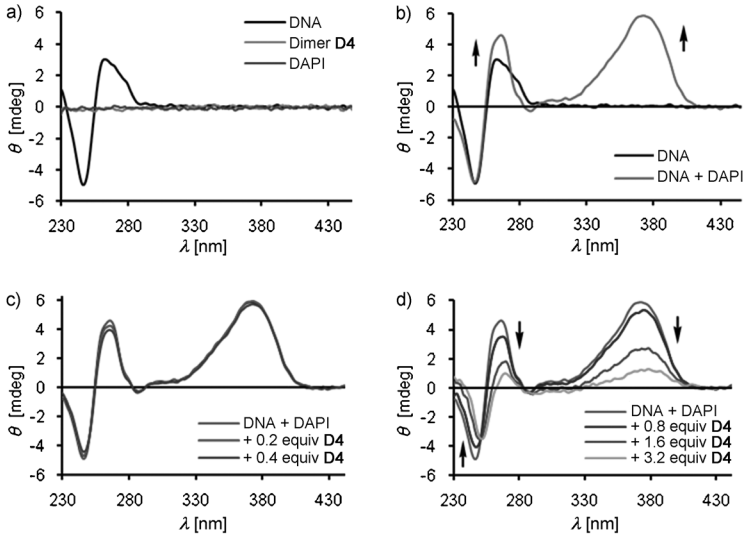


Figure 5. DAPI displacement measured by CD spectroscopy: a) CD signature of the parent oligonucleotide A_nT_n (0.5 μM in base pairs); b) Induced CD band at 376 nm from added DAPI (0.6 equiv. = 0.3 μM in base pairs); c) Negligible changes on addition of a stoichiometric amount of **D4** (0.4 equiv in base pairs); d) Rapid dye displacement by excess **D4**. All dimer equivalents are calculated per base pairs.

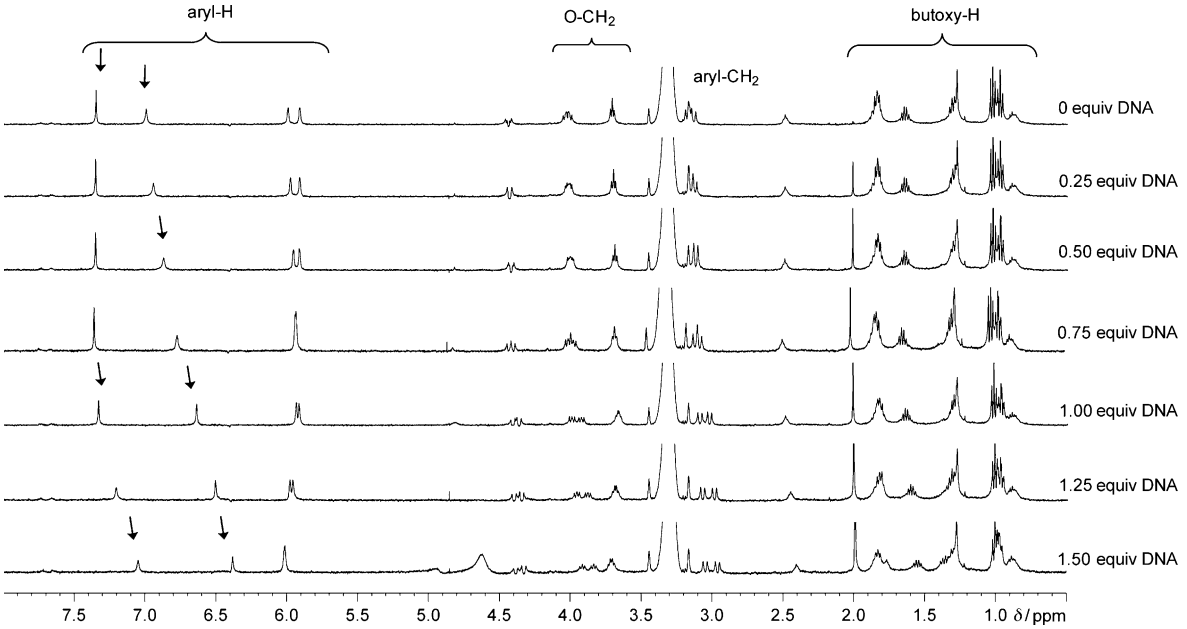


Figure 6. ^{1}H NMR spectra (500 MHz, CD_3OD/D_2O, 9:1, water suppression using excitation sculpting, 25 °C)[44] of dimer **D1** displaying the complexation-induced shift changes (arrows) on titration with the Dickerson dodecamer (concentration **D1**: 0.1 mM; increasing increments of DNA in 0.25 equiv steps from top to bottom; 1.0 equiv is here defined as the DNA concentration at which saturation is reached (equal to 0.08 molar equivalents of DNA; stoichiometry = 12:1).

tween the ammonium ions and N,O-heteroatoms of the nucleic bases agree well with these chemical shift changes of aromatic CH protons.

As DNA concentrations were increased, substantial line broadening of the signals occurred for the calixarene hydrogen atoms, but, again, mainly in the aromatic region and not in the butoxy tails, the methyl signal of which remained sharp. Most likely, this is a dynamic effect that must be interpreted in terms of restricted mobility of the aromatic head groups of the calixarenes inside the major groove, as opposed to the butoxy tails, which point away from the DNA molecule.

Both signal shifts and line broadening are valuable pieces of information on the orientation of the ligands inside the DNA "host". It might be argued that the postulated binding mode is counterintuitive, because an amphiphilic ligand should rather occupy the less polar groove with its nonpolar tails and present the charged ammonium groups into the bulk water. However, the center of the negative charge density inside the polyanionic double helix is located deep within both grooves, right above the nucleic bases.[43] All known rod-like groove-binders are therefore drawn into the grooves at their cationic ends. The butoxy tails and benzene back sides on the other hand may serve as hydrophobic contact areas for surrounding calixarene ligands, which form loose aggregates to optimally fill the spacious major groove of the DNA and still remain flexible/mobile.

Molecular modeling: Force-field calculations strongly support the accumulated experimental pieces of evidence detailed above. The calixarene dimers were docked into the major groove in various orientations, but only the orientation described above led to thermodynamically stable complex structures.[45] Monte-Carlo simulations were carried out for 1:1 complexes between calixarene dimer and double-stranded oligonucleotides (20 BP), employing MacroModel 9.7 (water, GB/SA solvation model, OPLS 2005 force-field, no constraints).[46] Figure 7 depicts two selected lowest energy conformations; in all structures, the ammonium and guanidinium moieties of the ligand form a network of hydrogen bonds with acceptors at the triple helix formation sites of the DNA base pairs and the butoxy tails point away from the DNA molecule. Ammonium dimers **D1** and **D3** bridge up to six BPs and form hydrogen bonds with their nucleic bases. The extended cationic fingers of the guanidinium dimers **D2** and **D4** bridge up to nine BPs. They even span the whole major groove and reach the opposite phosphodiester backbone anions; these additional interactions explain their superior DNA affinities. Intriguingly, the four-carbon alkyl bridge between both calix[4]arene heads is located 3–4 Å from the major groove floor and runs across two (**D1**/**D3**) or four BPs (**D2**/**D4**), respectively—an ideal situation for base recognition. Moreover, additional ammonium substituents attached to the alkyl bridge are predicted to further establish favorable hydrogen-bond contacts to DNA bases and phosphates.

Proliferation study: To investigate potential effects of the new DNA binders on the proliferation of living cells, four different human tumor cell lines were cultured: MCF-7 (breast carcinoma), SW 620 (colon carcinoma), HCT 116 (colon carcinoma), and H 460 (lung carcinoma). After 72 h incubation with monomeric and dimeric calixarenes, the cell growth rate was evaluated by performing an MTT assay,

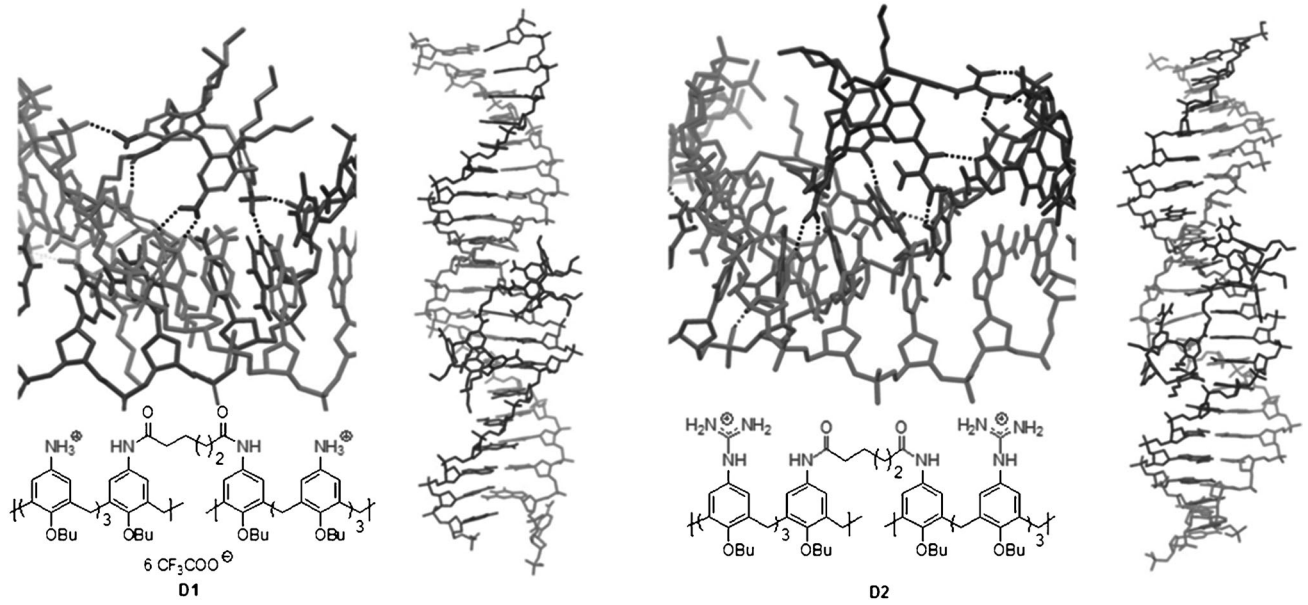


Figure 7. Calculated structures for dimers **D1** and **D2** with 20 BP ds DNA (MacroModel 9.7, OPLS, water, GB/SA).

which detects dehydrogenase activity in viable cells. Table 5 summarizes the results, as expressed in GI_{50} values.[47] In general, antiproliferative activities are rather high, with GI_{50} values in the low micromolar region. These seem to be correlated to the number of charges and/or solubility in cell culture medium and roughly track K_d values; monomers and dimers of the benzylamine series were equally effective. All calixarenes were prepared as clear dimethyl sulfoxide (DMSO) stock solutions, but precipitated, in part, when added to the cell culture medium. This was especially critical for dimer **D1**, with only two positive net charges. Thus, all results probably originate from effective ligand concentrations, which are much lower than calculated.

In spite of these promising data, they must be interpreted with care: cell growth inhibition might originate from DNA complexation inside the tumor cells, but it has not yet been firmly established that the cationic calixarenes pass the cell membranes. An alternative explanation for the antiproliferative effect cannot be ruled out: as amphiphilic molecules, the dimeric (and monomeric) calixarenes may self-assemble inside the cell membrane, and alter membrane potential as well as ion flux across the double layer, because the calixarene head groups are known to interact with various metal cations and anions.[48] Membrane potential measurements along with cell cycle analyses will shed more light on this aspect.[49]

Table 5. GI_{50} values [μM] of selected calixarene monomers and dimers on human tumor cell lines.

Calixarene	Cell lines groove depth [Å]			
	HCT 116	SW 620	MCF-7	H 460
D1	>100	>100	>100	>100
D2	95	>100	ppt.[a]	ppt.[a]
M3	2	2	2	2
D3	3	4	5	2
M4	2	2	2	2
D4	14	9	3	4

[a] Precipitation occurred.

SERS experiments and ITC measurements: These two experimental techniques were employed to further investigate the complexation behavior of the new class of dimeric calixarenes. Unfortunately, no SERS spectrum could be obtained from mixtures of monomer or dimer A and silver colloids, most likely because the butoxy tails do not bind to the surface of the silver nanoparticles.[50] Likewise, heat changes remained negligible for various calixarene/oligonucleotide complex pairs in microcalorimetric measurements.[51]

Conclusions and Outlook

This investigation focused on elucidating the binding mechanism of a new family of calixarene dimers. Although direct information from a crystal or NMR structure is not yet available, the synopsis of established DNA binding assays carried out with eight representative monomeric and dimeric calix[4]arenes strongly supports major-groove binding with hydrogen bonds between the cationic moieties on the upper rim of the calixarenes and nucleic bases as well as phosphate groups of the oligonucleotides. Because the majority of all the collected experimental binding data for the cationic calixarene monomers are largely consistent with those of the respective dimers (with the exception of those data typical for their spherical shape), it seems likely that these also prefer the major groove of double-stranded nucleic acids. We intend to replace the simple alkyl bridge between both calixarenes by a fragment that is able to recognize the base sequence of the spanned DNA region. The ultimate goal behind this work is control of gene expression by blocking specific promoter sequences. However, on the

way, modified dimeric calixarenes may also evolve as diagnostic tools and cytotoxic agents for tumor therapy.

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