Interactions of Antiviral Indolo[2,3-*b*]quinoxaline Derivatives with DNA

L. Marcus Wilhelmsson[1], *Ngarita Kingi*[2,3], *Jan Bergman*[2,*]

[1] Department of Chemical and Biological Engineering/Physical Chemistry, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

[2] Unit for Organic Chemistry, Department of Biosciences and Nutrition, Karolinska Institute, SE-14157, Huddinge, Sweden

[3] Drug Development, Vironova AB, Smedjegatan 6, SE-13134 Nacka, Sweden

[*] To whom correspondence should be addressed. email: jan.bergman@biosci.ki.se; tel.: +46 (0)8 608 92 04; fax: +46 (0)8 608 15 01

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Abbreviations: 11,11'-bidppz, 11,11'-bis(dipyrido[3,2-a:2',3'-c]phenazinyl); B220, 2,3 dimethyl-6(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (by **B**ergman- the **220**th synthesis in this project); C4(cpdppz)2, N,N' bis(cpdppz)-1,4-diaminobutane; CMV, cytomegalovirus; cpdppz, 12-cyano-12,13-dihydro-11H-8-cyclopenta[b]dipyrido[3,2-h:2',3'-j]phenazine-12-carbonyl; ct-DNA, calf thymus DNA; HCMV, human cytomegalovirus; HSV-1, herpes simplex type 1; LD, linear dichroism; LDr, reduced linear dichroism; MQ-water, MilliQ water; MS, multiple sclerosis; phen, 1,10-phenanthroline; poly(dAdT)2, duplex of two alternating adenine-thymine polynucleotides; RA, rheumatoid arthritis;

SAR, structure activity relationship; SDS, sodium dodecyl sulphate; VZV, varicella-zoster virus; YO, oxazole yellow; YOYO, homodimeric derivative of oxazole yellow (YO).

Abstract

Here we present the synthesis of five novel indoloquinoxaline derivatives and investigate the DNA binding properties of these monomeric as well as dimeric compounds using absorption, fluorescence and linear dichroism. Several of the mono- and di-cationic derivatives presented have previously demonstrated an excellent antiviral effect that is higher than already acknowledged agents against human cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV). We find that the DNA binding constants of the monomeric and dimeric derivatives are high ($\sim 10^6$) and very high ($\sim 10^9$), respectively. Results from the spectroscopic measurements show that the planar aromatic indoloquinoxaline moieties upon interaction with DNA intercalate between the nucleobases. Furthermore, we use poly(dA-dT)$_2$ and calf thymus DNA in a competitive binding experiment to show that all our derivatives have an AT-region preference. The findings are important in the understanding of the antiviral effect of these derivatives and give invaluable information for the future optimization of the DNA binding properties of this kind of drugs.

Introduction

The indole alkaloid ellipticine (**1** in Figure 1), first isolated in 1959 by Goodwin *et al.*[1] from *Ochrosia elliptica*, appeared to display anticancer activity and within the next two decades intense research in both the synthetic and biological areas of ellipticine and its analogues produced commercial cancer chemotherapy agents such as celiptium[2]. Unfortunately problems with the toxicity of these agents have more or less limited the success of these drugs in the pharmaceutical market.

Nevertheless research continued with the indolo[2,3-b]quinoxaline system (*i.e.* **2** in Figure 1) as analogues to the cytotoxic agent ellipticine. The replacement of the methyl groups in position 5 and 11 by nitrogen atoms gave little or no cytotoxicity against most cancer models except for Burkitt's lymphoma, a cancer type that is closely related to viral activity.[3] This information resulted in the development of 2,3-dimethyl-6(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (B-220)[4] (**3** in Figure 1) and further studies revealed that it exhibited good antiviral activity against herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV) and varicella-zoster virus (VZV). It has been reported that the mechanism of the antiviral action against HSV-1 appears to involve binding by intercalation into the DNA helix and, thus, disturbing the processes that are vital for viral uncoating.[5, 6] Further development of a new series of indoloquinoxaline derivatives related to B-220 have shown to have a significant effect against autoimmune inflammatory diseases such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) and other diseases.[7]

To improve DNA-binding of the family of ellipticine analogues and to optimize their antiviral effect we have synthesized a series of novel indoloquinoxaline derivatives (**4-8** in Figure 2). In this optimization of the drugs we have varied parameters that from previous studies are well-known to be important for DNA-binding *e.g.* the number of positive charges,[8-11] the number of interacting units (comparison between monomeric or dimeric compounds),[12-32] and the hydrophobicity of the interacting unit (see for example Karlsson *et al.*[33]). The quaternary mono- or di-cationic agents (**6-8** in Figure 2) developed using the structurally related **1-3** as parent compounds have indeed been shown to have

excellent inhibitory effects against human CMV (HCMV), HSV-1 and VZV. Modified inhibitory tests of antiviral activity against HCMV were compared with already acknowledged antiviral agents ganciclovir, phosphonomethanoic acid and the B-220 compound. This comparison indicated 100% inhibition by the novel compounds compared to 30, 50 and 20% inhibition of the acknowledged antiviral agents, respectively. These preliminary results are not conclusive as to what the underlying antiviral mechanism is although it appears to involve the impairment of the tegument protein that binds to the viral capsid. The novel compounds did not show any toxicity as assayed by propidium iodide staining of cell cultures of infected and uninfected human lung fibroblasts. [34] Obviously, further tests were considered necessary to comprehend the surprisingly different viral inhibition results especially exhibited between the novel compounds and B-220.

Here we perform extensive spectroscopic studies to investigate the interaction between the novel indoloquinoxaline derivatives (**4-8**) and DNA by determining the binding constant, the binding geometry and the AT-specificity. The variation in the structure, such as in the linker length and its position, the permanent/non-permanent quaternary nitrogen salt and the introduction of new substituent groups on the indoloquinoxaline moiety, are shown to have an immediate significant influence on the interaction between the ligands and DNA. These structure-activity relationships (SAR) give important understanding in the optimization of the antiviral effects of these promising drugs.

Chemistry

Indolo[2,3-*b*]quinoxaline (**2**, Figure 6) and its derivative (**2a**, Figure 6) were easily prepared by condensation of isatin and the appropriate 1,2-phenylenediamine in glacial acetic acid according to Schunck's and Marchlewski's method.[45, 46] Alkylation of these compounds was achieved when added to a solution containing 1-chloro-2-dimethylaminoethane hydrochloride and K_2CO_3 in acetone and refluxed for 24 h. Compound **4** was obtained by dissolving the free base (**3a**, Figure 6) in acetone and precipitation was induced by introduction of concentrated HCl. The amino compound **5** was prepared via a two-step synthesis starting with addition of one equivalent of potassium nitrate in sulfuric acid to

compound **3a** (Figure 6) to give 2,3-dimethyl-6-(2-dimethylaminoethyl-9-nitro-*6H*-indolo[2,3-*b*]quinoxaline. Reduction of the nitro group was achieved by catalytic hydrogenation of a suspension in N,N-dimethylacetamide using Pd/C under hydrogen pressure (2.7 atm) for 24 h. Finally hydrochloric acid was added, to a warm solution of 9-amino-2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-b]quinoxaline in water, until reaching a pH of 5. Compound **6** was prepared by refluxing compound **3a** (Figure 6) in acetonitrile with the addition of methyl iodide. The general procedure for synthesis of compounds **7**, **8** comprised of reflux of two equivalents of compound **3** (Figure 6) and one equivalent of a suitable α,ω-dibromoalkane in acetonitrile.

Results and Discussion

Table 1 shows the wavelength of maximum absorption of the low energy band and the corresponding extinction coefficient of the indoloquinoxaline derivatives (**4**-**8**) measured in MQ-water. Furthermore, the appearance of the lowest energy part of the absorption spectra of the indoloquinoxaline derivatives can be seen as the solid lines in Figure 3. The extinction coefficients are all reasonably similar ranging from 15000 to 21500 M^{-1} cm^{-1}. The low energy absorption maxima range from 345 to 374 nm with the monomers at slightly longer wavelengths (363-374 nm) than the dimers (345 nm). It is worth noting that the monomers containing exactly the same chromophoric unit, **4** and **6**, as expected has very similar wavelength maxima and extinction coefficient. The slightly lower extinction coefficient and absorption at longer wavelengths for monomer **5** compared to **4** and **6** can be explained by the addition of the amino-group in the 9-position of the indoloquinoxaline system. Also, it is worth noting that the extinction coefficients and wavelength maxima for **4**-**6** show great resemblance with previously reported values for molecules built from the same kind of chromophore even though those measurements were performed in ethyl acetate.[35] As expected, also dimers **7** and **8** containing the same chromophoric unit have very similar wavelength maxima and extinction coefficients. The fact that **7** and **8** which have two chromophoric units that are essentially identical to monomers **4** and **6** do not exhibit a twofold increase

in extinction coefficient can be explained by stacking of the two chromophores within the dimers. This stacking results in an excitonic interaction between the two chromophores of the dimeric compounds and hence a considerable change in spectral properties. Also the change in absorption maxima from 363 nm in the monomers to 345 nm in the dimers comes as a result of this excitonic interaction.

Table 2 shows the estimated equilibrium constants of binding of **4**-**8** to poly(dA-dT)$_2$ as well as the ratio between the binding constant when the same drugs interacts with poly(dA-dT)$_2$ and ct-DNA. The binding constants of the monomeric indoloquinoxaline derivatives (**4**-**6**) to poly(dA-dT)$_2$ are in the order of 10^6. Compound **6**, which has exactly the same aromatic ring system as **4**, has a slightly higher equilibrium constant. Consequently, the difference in binding constant has to be due to the higher hydrophobicity of **6** as an effect of the additional methyl on the attached aminoethyl group. In the case of compound **5** the slight increase in DNA binding constant compared to compound **4** has to be ascribed to the addition of the amino-group on the aromatic ring system. As can be seen in the Table the binding constants of compounds (**7**-**8**) to poly(dA-dT)$_2$ are considerably higher (10^8-10^9). This is also expected since these dimeric compounds have both double the amount of functional groups and positive charges compared to **4**-**6**.

A general conclusion about the drugs that can be drawn from the measurements is that they all prefer AT-DNA. As can be seen in Table 2 the preference for poly(dA-dT)$_2$ compared to ct-DNA range from 1.8 to 3.0. Since ct-DNA consists of a mixture of sites with different base composition and, thus, different binding constants and also the number of accessible drug binding sites in ct-DNA compared to in poly(dA-dT)$_2$ is difficult to estimate, the actual values of AT-preference should not be over-interpreted. However, as a consequence of the general AT-preference among the five drugs and the magnitude of the $K_{AT}/K_{ct\text{-}DNA}$ there is no doubt that all the indoloquinoxaline derivatives, like their parent compounds,[36-38] prefer to bind to AT-DNA (AT-regions).

Figure 3 shows the absorption spectra of **4**-**8** in presence and absence of ct-DNA. As can be seen in the figure the general trend is that the molecules show a considerable hypochromic effect and a

significant red-shift of the absorption maxima upon addition of ct-DNA (dashed lines). These are common effects when drugs bind to DNA by either intercalation or groove binding. The large hypochromic effect (14-34%) and red-shift (6-19 nm) displayed by these drugs indicate intercalation rather than groove binding (we will show more distinctive evidence of intercalation below). The larger red-shift observed for the dimers (Figure 3 d-e) can be explained by the decrease in excitonic effects as the dimers unstack upon their binding to the DNA.

In Figure 4 the fluorescence spectra of **4**, **6**, **7**, and **8** both in presence and in absence of poly(dA-dT)$_2$ are presented. As an effect of the lack of emission both with and without DNA, the result from the fluorescence measurements of compounds **5** is not shown in the figure. Since the only difference in the chromophoric unit in **5** compared to **4** and **6** is the amino group in the 9-position, this group has to be the reason for the quenching of the emission in **5**. As can be seen in Figure 4 the four fluorescent drugs all have an intrinsic emission centered at 515 nm in buffered solution (solid lines). Furthermore, upon binding to DNA they all exhibit a considerable increase in emission (dashed lines) as well as a blue-shift of more than 30 nm. Increase in emission upon binding to DNA is often considered to be a good indication of intercalation of the drug between the base pairs of the DNA and, thus, these measurements further support an intercalative mode of binding.

Figure 5 shows the flow linear dichroism (LD) spectra of the five drugs, **4**-**8**, in presence of ct-DNA. As can be seen from the figure the LD of the bound drugs is purely negative. In the region where DNA has no influence on the signal (> 300 nm), the LD spectra are virtually perfect mirror images of the corresponding absorption spectra of the DNA-bound drugs (for comparison see Figure 3). A negative LD proves that the angle between the DNA helix axis and the planar chromophore unit(s) of the drugs is larger than the magic angle (54.7°). When calculating the LD^r of the band originating from the five drugs and subsequently the angles between the DNA helix axis and the planar chromophore unit(s) they are all in the range between 70 and 90°. This is indeed good evidence for an intercalative mode of binding for all the five drugs. After determination of the binding mode by LD, SDS was added

to all six DNA-drug solutions. The LD-signal of the drugs disappeared immediately leaving only a pure DNA-LD centered around 260 nm. This confirms the non-covalent nature of the DNA-interaction of these drugs and indicates a fast DNA-dissociation process.

The very promising antiviral effect exhibited by some of the indoloquinoxaline derivatives presented here makes them interesting to investigate in terms of their DNA binding properties. Moreover, it is important to understand the structure-activity relationship (SAR) within this set of indoloquinoxalines not only for the current derivatives and possible use of them as drugs but also for their future structural modification and further optimization.

The results obtained from absorption, fluorescence and LD measurements all give evidence of a mode of binding where the planar aromatic parts of the indoloquinoxaline derivates are non-covalently intercalated between the basepairs of the DNA. This mode of binding has previously been reported for the parent compounds (9-OH-)B220[39, 40] and is very common for positively charged planar aromatic molecules like *e.g.* oxazole yellow (YO)[15-17] and ethidium[41]. For the dimers (**7**-**8**) this means that they are bis-intercalators with the interconnecting linker ending up in one of the grooves of the DNA. From the present measurements we cannot conclude whether the linkers are situated in the minor or major groove or if they are equally distributed in the two grooves. For the structurally related bis-intercalator YOYO as well as for the naturally occurring bis-intercalator echinomycin, however, the linker interconnecting the monomeric units has been found to reside in the minor groove,[19, 42] suggesting that the linkers of our indoloquinoxaline dimers may have a minor groove preference. Also, earlier NMR studies on related compounds suggest that the side chain resides in the minor groove.[36] For the dimers (**7**-**8**) it is also important to identify the size of the DNA binding site. However, with the data obtained in this investigation it is not possible to directly determine this number. Nevertheless, from the length of the linkers and the overall structures of the dimers we estimate the binding site size to be four and three basepairs for **7** and **8**, respectively. The smaller binding site size of **8** is further supported by the fact that

using this binding site size in the calculation of the equilibrium constant (see Table 2) results in a similar DNA binding constant to that of **7**, which we expect due to the high structural resemblance of the two dimers. Finally, it should be noted that the high similarity in the results for all derivatives presented here demonstrates that the structural changes that we introduce to the indoloquinoxaline planar system only have minor effects on the mode of binding of these drugs.

The DNA-binding constant is a very important property for an antiviral drug. In the results obtained here we find that the monomeric indoloquinoxaline derivates (**4**-**6**) have DNA-binding constants (AT sequence) of approximately 10^6. DNA binding constants in the order of 10^6, at comparable or lower ionic strengths and similar temperatures, have previously been reported for familiar intercalators like ethidium,[43] YO,[16] actinomycin D,[44] daunomycin,[43] and $[Ru(phen)_2dppz]^{2+}$[26]. These common intercalators are successfully used or have been proposed to be used for example as DNA stains or as antibiotics or anticancer therapeutics and, consequently, the indoloquinoxaline monomers (**4**-**6**) should constitute very promising DNA binding drugs. For the dimeric indoloquinoxaline derivates (**7**-**8**) we determine DNA binding constants of 10^8-10^9 (binding constant of **8** would be even higher if the same binding site size was used as for **7**; see Table 2). Previously, binding constant estimates of other dimeric DNA-ligands like YOYO ($K\approx10^{10}$-10^{12}),[16] [μ-C4$(cpdppz)_2(phen)_4Ru_2]^{4+}$ ($K\approx10^9$),[28] and [μ-(11,11'-bidppz)$(phen)_4Ru_2]^{4+}$ ($K\approx10^{12}$)[29] as well as for larger DNA-ligands like distamycin ($K\approx10^9$)[43] and netropsin ($K\approx10^9$)[43] have been performed at comparable or lower ionic strengths and similar temperatures. When comparing these values to the ones obtained here for **7** and **8** one has to remember that for the previously reported dimers the charge is two times higher and that for one of them, [μ-(11,11'-bidppz)$(phen)_4Ru_2]^{4+}$,[29] the measurements are performed at only 10 mM Na^+. Taking this into consideration both **7** and **8** have a remarkably high binding constant to DNA, which could explain the high antiviral activity of them compared to the already acknowledged ganciclovir, phosphonomethanoic acid and the B-220 compound.

In conclusion, the novel indoloquinoxaline derivatives (**4**-**8**) investigated here comprise a group

of very promising antiviral agents that bind strongly but non-covalently to DNA in an intercalative mode. They are found to have equally high binding constants as already established DNA drugs and dyes and show a preference for AT-regions. The AT-specificity, a property shared with some of the DNA drugs and dyes mentioned above, is potentially useful for targeting viral genomes that are especially AT-rich.

Experimental Section

Melting points were determined on a Leica Kofler hot stage and are uncorrected. NMR data were recorded on a Bruker DPX at 300.1 MHz for ^{1}H and 75.5 MHz for ^{13}C, respectively. IR spectra were acquired on a Perkin-Elmer FT-IR 1600 spectrophotometer. Elemental analyses were performed by Mikroanalytisches Laboratorium KOLBE, Germany and were within ± 0.4% of the calculated values

General procedure for indolo[2,3-*b*]quinoxalines.

6H-Indolo[2,3-*b*]quinoxaline **(2)** and 2,3-dimethyl-*6H*-indolo[2,3-*b*]quinoxaline **(2a)** were prepared on a 0.10 molar scale according to Schunck´s and Marchewski´s method.[45, 46] Compounds **2** or **2a** (1 eq.) was added to a solution of 1-chloro-2-dimethylaminoethane hydrochloride (1.2 eq.), K_2CO_3 (10 eq.) in acetone and refluxed 24 h, when the reaction mixture was poured into water, the solid thus formed was isolated by filtration.

6-(2-Dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline (3).

Yield 76%; mp 95-97 °C (lit.,[47] 88-90 °C); ^{1}H-NMR ($CDCl_3$) δ 8.47 (d, 1H, *J*=7.7 Hz), 8.30 (dd, 1H, *J*=1.2, *J*=8.8 Hz), 8.12 (dd, 1H, *J*=1.4, *J*=8.4 Hz), 7.76-7.80 (m, 4H), 7.37-7.43 (m, 1H), 4.80 (t, 2H, *J*=7.10 Hz), 3.14 (t, 2H, *J*=6.45 Hz), 2.60 (s, 6H); ^{13}C-NMR ($CDCl_3$) δ 145.0 (s), 143.6 (s), 140.0 (s), 139.7 (s), 139.0 (s), 130.9 (d), 129.0 (d), 128.4 (d), 127.3 (d), 125.7 (d), 122.3 (d), 120.8 (d), 119.2 (s), 109.3 (d), 55.8 (t), 44.4 (q), 38.3 (t); IR (neat) υ_{max}: 3059, 2974, 2946, 2822, 2767, 1581, 1466, 1406, 742 cm^{-1}.

2,3-Dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline (3a).

Yield 72%; mp 134-136 °C; ^{1}H-NMR ($CDCl_3$) δ 8.42 (d, 1H, *J*=7.72 Hz), 8.02 (s, 1H,), 7.86 (s, 1H), 7.67-7.69 (m, 2H), 7.34-7.39 (m, 1H), 4.80 (t, 2H, *J*=7.3 Hz), 3.17 (t, 2H, *J*=7.1 Hz), 2.62 (s, 6H), 2.52 (s, 6H); ^{13}C-NMR ($CDCl_3$) δ 144.8 (s), 143.1 (s), 139.0 (s), 138.7 (s), 138.7 (s), 137.9 (s), 135.9 (s), 130.4 (d), 128.1 (d), 126.5 (d), 122.0 (d), 120.7 (d), 119.4 (s), 109.2 (d), 55.6 (t), 44.2 (q), 38.0 (t), 20.0 (q), 19.7 (q); IR (neat) υ_{max}: 3433, 2954, 2830, 2780, 1581, 1488, 1467, 1404, 1349, 1217, 1165 cm^{-1}.

2,3-Dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline hydrochloride (4).

Concentrated hydrochloric acid was added to a heated solution of compound **3a** (0.80 g, 2.5 mmol) in acetone (40 ml). The solid thus formed was isolated by filtration.

Yield 0.89 g (89%); mp 283-286°C; ^{1}H-NMR (DMSO-d_6) δ 10.88 (br s, NH), 8.35 (d, 1H, *J*=7.5 Hz), 8.00 (s, 1H), 7.95 (d, 1H, *J*=8.22 Hz), 7.86 (s, 1H), 7.76 (t, 1H, *J*=8.26 Hz), 7.42 (t, 1H, *J*=7.45 Hz), 4.89 (t, 2H, *J*=6.56 Hz), 3.65-3.60 (m, 2H), 2.93 (s, 3H), 2.92 (s, 3H), 2.51 (s, 3H), 2.50 (s, 3H); ^{13}C-NMR (DMSO-d_6) δ 145.0 (s), 143.3 (s), 139.4 (s), 138.5 (s), 138.4 (s), 137.4 (s), 136.2 (s), 131.0 (d), 127.8 (d), 126.6 (d), 122.1 (d), 121.3 (d), 119.2 (s), 110.6 (d), 53.9 (t), 42.3 (q), 36.4 (t), 20.0 (q), 19.7 (q); IR (neat) υ_{max}: 3504, 3287, 3214, 2987, 2514, 1923, 1609, 1519, 1471, 1227, 1130, 968, 861, 759, 604 cm^{-1}. Anal. calcd for ($C_{20}H_{23}ClN_4$•1/6H_2O) C, H, N.

9-Amino-2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline hydrochloride (5).

9-Amino-2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline (0.80 g, 2.62 mmol) was easily prepared according to Vallberg´s method,[39] heated in water (80 ml) with the addition of HCl (1 M) to a pH of 5. The hydrochloride was isolated by removing solvent by rotary evaporation.

Yield 0.84 g (94%); mp 275-278°C; ^{1}H-NMR (DMSO-d_6) δ 9.78 (br s, NH), 7.99 (s, 1H), 7.82 (s, 1H), 7.59 (s, 1H), 7.56-7.54 (m, 1H), 7.08 (dd, 1H, *J*=8.59, *J*=2.31 Hz), 5.32 (br s, 2H, NH_2), 4.75 (t, 2H, *J*=6.00 Hz), 3.58 (br s, 2H), 2.92 (s, 6H), 2.51 (s, 3H), 2.50 (s, 3H); ^{13}C-NMR (DMSO-d_6) δ 145.4 (s), 143.6 (s), 139.2 (s), 138.9 (s), 138.3 (s), 137.3 (s), 135.5 (s), 135.4 (s), 128.1 (d), 126.4 (d), 120.1 (s), 118.9 (d), 110.8 (d), 105.7 (d), 54.6 (t), 42.6 (q), 36.5 (t), 20.0 (q), 19.7 (q); IR (neat) υ_{max}: 3371, 2958,

2613, 1493, 1479, 1213, 528, 715 cm^{-1}. Anal. calcd for ($C_{20}H_{24}ClN_5$•1/6H_2O) C, H, N.

2,3-Dimethyl-6-[2-(trimethylamino)ethyl]-*6H*-indolo[2,3-*b*]quinoxaline iodide (6).

Methyl iodide (0.32 ml, 5.2 mmol) was added to a solution of compound **3a** (1.60 g, 5.0 mmol) in acetonitrile (50 ml). The solid thus formed was isolated by filtration.

Yield 1.83 g (80%); mp 281-283°C; ^{1}H-NMR (DMSO-d_6) δ 8.36 (d, 1H, *J*=7.52 Hz), 8.02 (s, 1H), 7.90-7.83 (m, 2H), 7.80 (t, 1H, *J*=7.22 Hz), 7.45 (t, 1H, *J*=7.46 Hz), 4.96 (t, 2H, *J*=6.94 Hz), 3.89 (t, 2H, *J*=6.99 Hz), 3.28 (s, 9H), 2.49 (s, 3H), 2.48 (s, 3H); ^{13}C-NMR (DMSO-d_6) δ 144.5 (s), 143.0 (s), 139.6 (s), 138.5 (s), 138.4 (s), 137.9 (s), 136.4 (s), 131.0 (d), 128.1 (d), 126.7 (d), 122.1 (d), 121.5 (d), 119.2 (s), 110.6 (d), 61.9 (t), 52.7 (q), 35.3 (t), 20.0 (q), 19.7 (q); IR (neat) υ_{max}: 3402, 3010, 1586, 1489, 1469, 1405, 1352, 923, 745 cm^{-1}. Anal. calcd for ($C_{21}H_{25}IN_4$) C, H, N.

General procedure for bis-quaternary bromides.

6-[2-(Dimethylamino)ethyl]-*6H*-indolo[2,3-*b*]quinoxaline was prepared according to the general procedure described for compound **3**. The free base (2 eq.) was added to a solution of the appropriate α,ω-dibromohexane (1 eq.) in acetonitrile and refluxed for 24 h. The solid thus formed was isolated by filtration.

Bis-indolo-(2,3-*b*)quinoxaline-*6H*-(3,3;10,10-tetramethyl)-3,10-di-(3,10)-azadodecane bromide (7).

Yield 72%; mp 260-262°C; ^{1}H-NMR (DMSO-d_6) δ 8.42 (d, 1H, *J*=7.6 Hz), 8.28 (d, 1H, *J*=8.3 Hz), 8.09 (d, 1H, *J*=8.4 Hz), 7.96 (d, 1H, *J*=8.1 Hz), 7.86-7.83 (m, 2H), 7.76-7.74 (m, 1H), 7.49 (t, 1H, *J*=7.5 Hz), 4.98 (br t, 2H, *J*=6.8 Hz), 3.85 (br t, 2H, *J*=6.8 Hz), 3.53-3.47 (m, 2H), 3.25 (s, 6H), 1.78 (br s, 2H), 1.30 (br s, 2H); ^{13}C-NMR (DMSO-d_6) δ 144.8 (s), 143.6 (s), 139.8 (s), 139.6 (s), 139.0 (s), 131.6 (d), 129.3 (d), 129.2 (d), 127.4 (d), 126.6 (d), 122.5 (d), 121.8 (d), 119.1 (s), 110.7 (d), 63.2 (t), 59.2 (t), 50.8 (q), 35.0 (t), 25.4 (t), 21.7 (t); IR (neat) υ_{max}: 3406, 3004, 2933, 1610, 1469, 1413, 1201, 1120, 769, 752 cm^{-1}.Anal. calcd for ($C_{42}H_{48}Br_2N_8$•1/4H_2O) C, H, N.

Bis-indolo-(2,3-*b*)quinoxaline-*6H*-(3,3;7,7-tetramethyl)-3,10-di-(3,7)-azaheptane bromide (8).

Yield 68%; mp 258-260°C; ^{1}H-NMR(DMSO-d_6) δ 8.38 (d, 1H, *J*=7.5 Hz), 8.25-8.24 (m, 1H), 8.08-

8.06 (m, 1H), 7.98 (d, 1H, *J*=8.2 Hz), 7.81-7.73 (m, 2H), 7.46 (t, 1H, *J*=7.4 Hz), 5.03 (br t, 2H, *J*=7.0 Hz), 3.96 (br t, 2H, *J*=7.1 Hz), 3.72 (br t, 2H, *J*=7.5 Hz), 3.38 (s, 6H), 2.45 (br s, 2H); ^{13}C-NMR (DMSO-d_6) δ 144.7 (s), 143.5 (s), 139.7 (s), 139.5 (s), 138.9 (s), 131.5 (d), 129.2 (d), 127.4 (d), 126.5 (d), 122.4 (d), 121.7 (d), 119.0 (s), 110.7 (d), 60.5 (t), 59.9 (t), 50.9 (q), 35.0 (t), 16.9 (t); IR (neat) υ_{max}: 3349, 3004, 1607, 1467, 1415, 1116, 938, 769, 752 cm^{-1}.Anal. calcd for ($C_{39}H_{42}Br_2N_8$•1/4H_2O) C, H, N.

Chemicals for DNA-binding Studies

Calf thymus (ct) DNA, obtained from Sigma, was dissolved in buffer and filtered twice through a 0.8 μm Millipore filter before use. The polynucleotide, poly(dA-dT)$_2$, was purchased from Amersham Biosciences and used as obtained. All experiments were performed in aqueous buffer (10 mM phosphate buffer at pH 7.5 and NaCl added to a total Na^+-concentration of 100 mM). Sodium dodecyl sulphate (SDS) was purchased from Sigma-Aldrich. The indoloquinoxaline derivatives were synthesized as described above.

Sample Preparation

Samples were prepared by mixing equal volumes of indoloquinoxaline derivatives and DNA dissolved in buffer. Unless otherwise stated the sample concentrations were 10 μM and 5 μM of the monomeric and dimeric indoloquinoxaline derivatives, respectively, and 160 μM ([base]) of DNA. Concentrations were determined on a Varian Cary 4B spectrophotometer. The extinction coefficients used for the DNAs were: ε_{260nm}=6600 M^{-1}cm^{-1} for ct-DNA and ε_{262nm}=6600 M^{-1}cm^{-1} for poly(dA-dT)$_2$. Extinction coefficient used for the indoloquinoxaline derivatives were determined as described below.

Extinction Coefficient Determination

The extinction coefficient of the indoloquinoxaline derivatives were determined by measuring the absorption of samples of known concentration. Samples were prepared by weighing out small amounts of the indoloquinoxaline derivatives, typically 2 mg, and dissolving them in known volumes of MQ water. Several absorption spectra of each sample were recorded on a Varian Cary 4000 spectrophotometer at room temperature and an average of the measurements was calculated. The

extinction coefficients were determined as an average of between three and five independent sample preparations.

Fluorescence

Emission spectra were recorded at room temperature on a Xenon lamp equipped SPEX fluorolog 3 spectrofluorimeter (JY Horiba) between 365 nm and 750 nm using an excitation wavelength of 360 nm. Concentrations of the indoloquinoxaline derivatives were kept low in order to avoid inner filter effects.

Flow Linear Dichroism

Linear dichroism (LD)[48] is defined as the difference in absorbance of linearly polarized light parallel and perpendicular to a macroscopic orientation axis (here the flow direction):

$$LD(\lambda) = A_{II}(\lambda) - A_{\perp}(\lambda) \tag{1}$$

The reduced linear dichroism LD^r is calculated as

$$LD^r(\lambda) = LD(\lambda)/A_{iso} \tag{2}$$

where A_{iso} represents the absorbance of the same isotropic sample. The LD^r of a single electronic transition, *i*,

$$LD^r{}_i = \frac{3}{2} S(3\cos^2 \alpha_i - 1) \tag{3}$$

is related to the angle α_i between the *i*:th transition moment direction and the molecular orientation axis, in this case the DNA helix axis. Samples with indoloquinoxaline derivatives and DNA were oriented in a Couette flow cell with an outer rotating cylinder at a shear gradient of approximately 3000 s^{-1}. LD spectra were measured on a Jasco J-720 CD spectropolarimeter equipped with an Oxley prism to obtain

linearly polarized light. All spectra were recorded between 200 nm and 650 nm and baseline-corrected by subtracting the spectrum recorded for the non-oriented sample.

Equilibrium Constant Determination

Buffered solutions containing 0, 25, 100, and 250 μM poly(dA-dT)$_2$ as well as 10 or 5 μM of the fluorescent monomeric or dimeric indoloquinoxaline derivatives **4**, **6**, **7**, and **8**, respectively, were prepared. Both absorption (measured on a Varian Cary 4000 spectrophotometer between 200 and 650 nm) and fluorescence spectra (for details see above) of the solutions were recorded at 22°C. Hereafter the solutions were diluted 10 and 100 times and their absorption and fluorescence spectra once again recorded. The fraction of bound molecules was estimated from the measured spectra and subsequently used to calculate the equilibrium constants of the DNA-binding. When calculating the equilibrium binding constants, the sizes of the DNA binding sites were assumed to be 2, 2, 4, and 3 base pairs for **4**, **6**, **7**, and **8**, respectively. The DNA binding site size for dimer **8** is assumed to be smaller due to the shorter linker between the two planar aromatic ring systems compared to that of dimer **7**.

For the virtually non-fluorescent compound **5**, competitive DNA binding with compound **6** was used to estimate the binding constant. Two buffered solutions containing 6.25 μM poly(dA-dT)$_2$ as well as 2.5 or 3.75 μM of **6** were prepared. Also, two solutions containing the same amount of poly(dA-dT)$_2$ and half the amount of **6** compared to the two above as well as 1.25 or 1.875 μM of **5**, respectively, were prepared. The fluorescence of DNA-bound **6** from the four samples was measured. The changes in emission as an effect of compound **5** competing with compound **6** about the DNA binding sites in combination with the previously determined DNA binding constant of **6** were then used to estimate the equilibrium binding constant of the non-fluorescent **5** when binding to DNA.

The difference in equilibrium constant of binding of the various indoloquinoxaline derivatives to the two forms of DNA, ct-DNA and poly(dA-dT)$_2$, was measured using flow LD. The LD of each indoloquinoxaline derivative (5 and 10 μM of the dimeric and monomeric ones, respectively) bound to ct-DNA (160 μM) was recorded and compared to the LD of the same molecule when mixed

into a solution of a combination of ct-DNA (80 µM) and poly(dA-dT)$_2$ (80 µM). As a result of the short polynucleotide strands (699 base pairs), the measured LD of the indoloquinoxaline derivatives bound to poly(dA-dT)$_2$ (160 µM) has virtually no intensity.

Figure Legends

Figure 1. The alkaloid ellipticine, **1**, the indolo[2,3-b]quinoxaline system, **2**, and the parent compound of the indoloquinoxaline derivatives used in this study: 2,3-dimethyl-6(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (B-220), **3**.

Figure 2. Novel monomeric and dimeric indoloquinoxaline derivatives synthesized and used in this study. **4** 2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline hydrochloride, **5** 9-amino-2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline hydrochloride, **6** 2,3-dimethyl-6-[2-(trimethylamino)ethyl]-*6H*-indolo[2,3-*b*]quinoxaline iodide, **7** bis-indolo-(2,3-*b*)quinoxaline-*6H*-(3,3;10,10-tetramethyl)-3,10-di-(3,10)-azadodecane bromide, and **8** bis-indolo-(2,3-*b*)quinoxaline-*6H*-(3,3;7,7-tetramethyl)-3,10-di-(3,7)-azaheptane bromide.

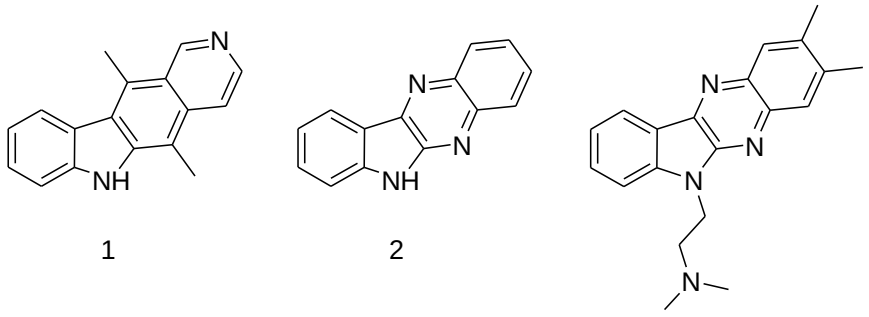
Figure 3. Absorption spectra of a) **4**, b) **5**, c) **6**, d) **7**, and e) **8** in presence (dashed lines) and absence (solid lines) of calf thymus DNA. Concentration of calf thymus DNA is 160 µM ([base]) and of the monomeric and dimeric indoloquinoxaline derivatives 10 and 5 µM, respectively. Measurements performed at room temperature in a 10 mM phosphate buffer, pH 7.5 (total Na^+-concentration 100 mM).

Figure 4. Emission spectra of a) **4**, b) **6**, c) **7**, and d) **8** in presence (dashed lines) and absence (solid lines) of poly(dA-dT)$_2$. Compound **5** is virtually non-fluorescent both in presence and absence of DNA and therefore not shown. Concentration of poly(dA-dT)$_2$ is 100 µM ([base]) and of the monomeric and dimeric indoloquinoxaline derivatives 10 and 5 µM, respectively. Measurements performed at room temperature in a 10 mM phosphate buffer, pH 7.5 (total Na^+-concentration 100 mM).

Figure 5. Flow linear dichroism spectra of **4** (black), **5** (red), **6** (green), **7** (blue), and **8** (cyan) in presence of calf thymus DNA. Concentration of calf thymus DNA is 160 µM ([base]) and of the monomeric and dimeric indoloquinoxaline derivatives 10 and 5 µM, respectively. Measurements performed at room temperature in a 10 mM phosphate buffer, pH 7.5 (total Na^+-concentration 100 mM).

Figure 6. Left: *6H*-indolo[2,3-*b*]quinoxaline **2** and 2,3-dimethyl-*6H*-indolo[2,3-*b*]quinoxaline **2a**. Right: 6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline **3** and 2,3-dimethyl-6-(2-dimethylaminoethyl)-*6H*-indolo[2,3-*b*]quinoxaline **3a**.

Figures



1 2 3

Figure 1.

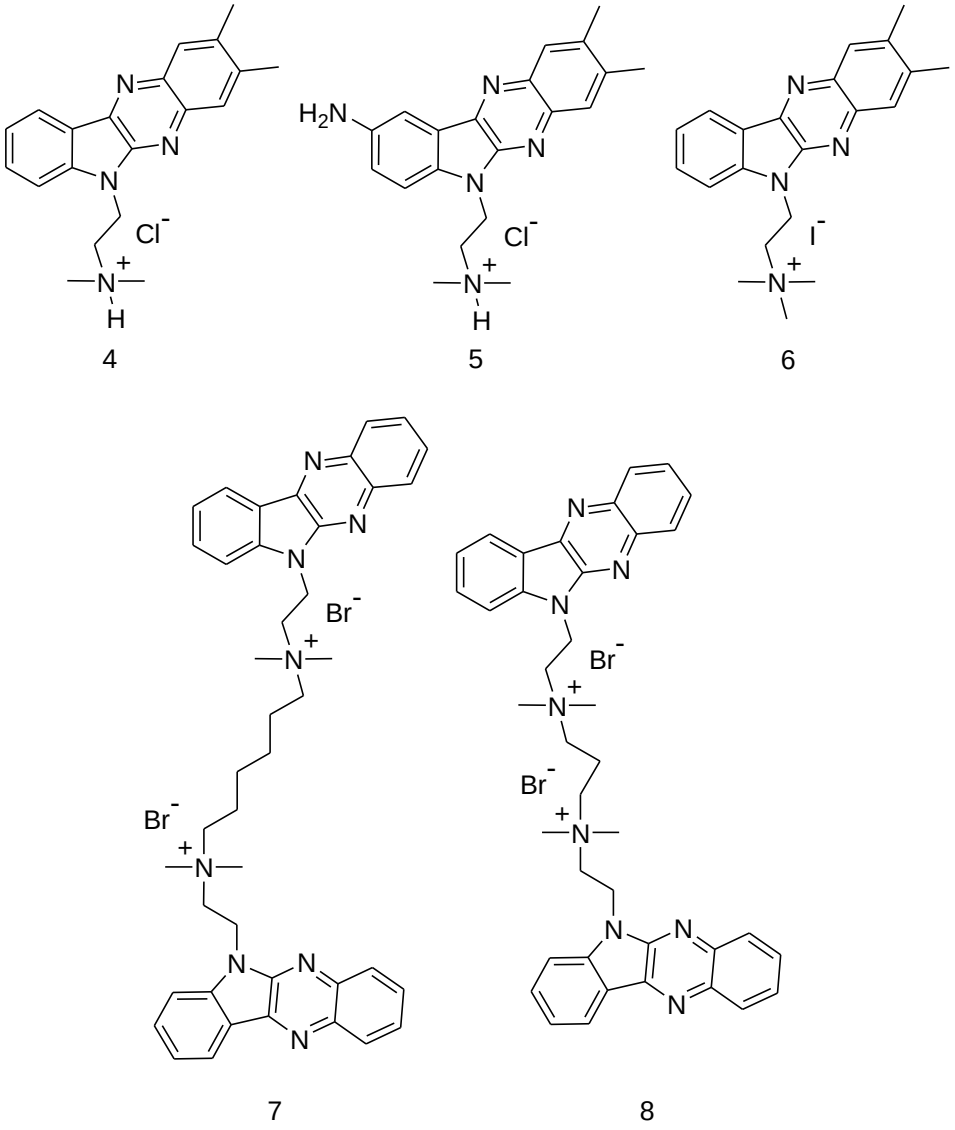


Figure 2.

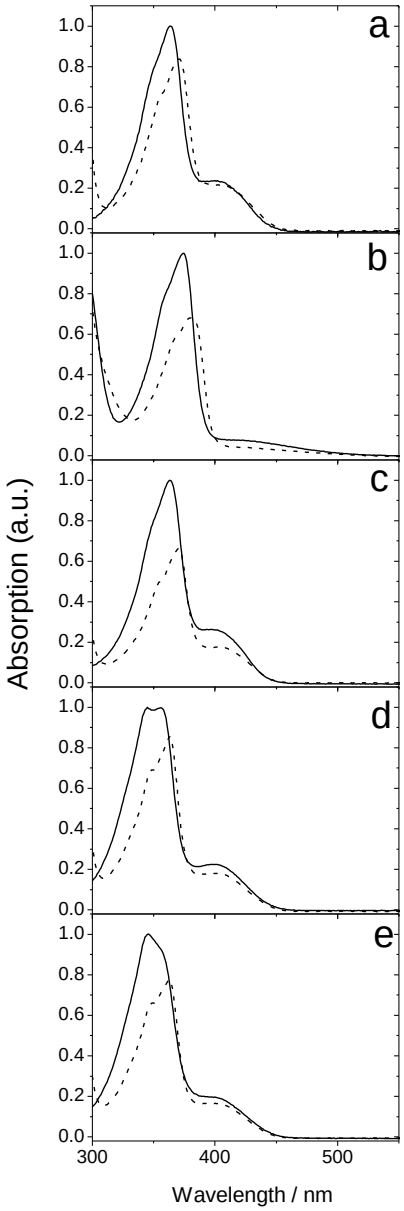

a
b
c
d
e
Absorption (a.u.)
Wavelength / nm
1.0
0.8
0.6
0.4
0.2
0.0
300
400
500

Figure 3.

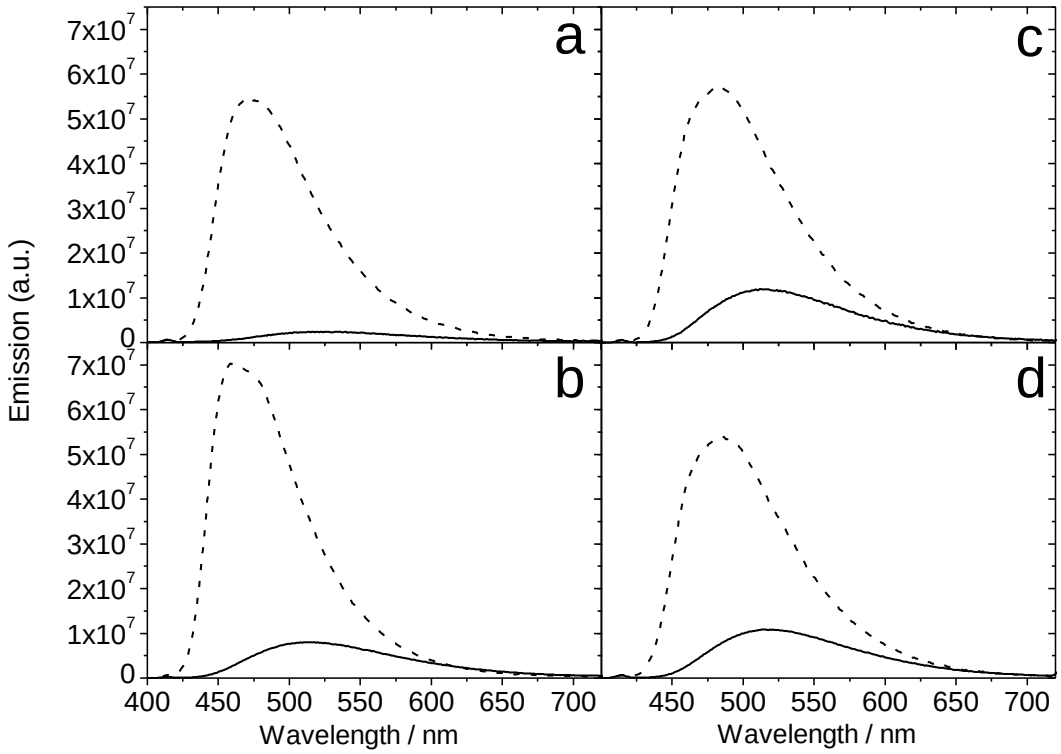


Figure 4.

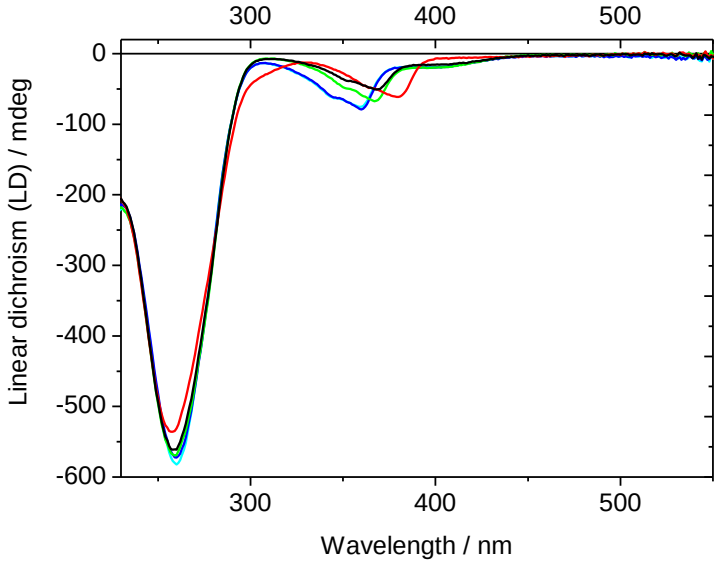


Figure 5.

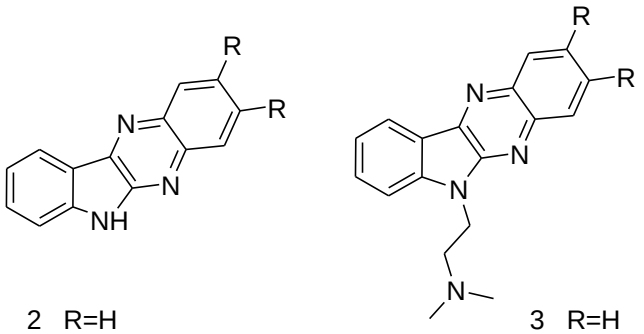


Figure 6.

Tables

Table 1. Extinction coefficients of the indoloquinoxaline derivatives (**4-8**) measured in MQ-water at room temperature.

Drug	λ_{max} / nm	ε / M^{-1} cm^{-1}
4	363	17500
5	374	15000
6	363	19000
7	345	21500
8	345	21000

Table 2. Equilibrium constants of the indoloquinoxaline derivatives (**4-8**) binding to poly(dA-dT)$_2$ and ratio in binding constants between poly(dA-dT)$_2$ and calf thymus DNA in a competitive binding experiment. Measurements performed at 22°C in a 10 mM phosphate buffer, pH 7.5 (total Na^+-concentration 100 mM).

Drug	K_{AT}	$K_{AT}/K_{ct\text{-}DNA}$
4	10^5-10^6	1.8
5	10^6-10^7 [a]	1.7
6	10^6-10^7	2.4
7	10^8-10^9	3.0
8	10^8-10^9 [b]	1.9

[a] Due to the lack of fluorescence of **5** its equilibrium constant of DNA binding was estimated in a competitive binding experiment with **6**.

[b] The equilibrium constant of DNA binding of **8** was calculated using a binding site size of 3 base pairs

(for the other dimer, **7**, a site size of 4 was used).

Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra of compounds **3**, **3a**, **4**, **5**, **6**, **7** and **8**. Elemental analysis results of target compounds **4**, **5**, **6**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Tables of Contents Graphic.