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Synthesis and DNA Binding Studies of a New Asymmetric Cyanine Dye Binding in the Minor Groove of [poly(dA-dT)]$_2$

H. Jonas Karlsson, Per Lincoln and Gunnar Westman*

Department of Chemistry and Bioscience, Chalmers University of Technology, Kemivägen 10, S-41296 Göteborg, Sweden

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Abstract—A new asymmetric cyanine dye has been synthesised and its interaction with different DNA has been investigated. In this dye, **BEBO**, the structure of the known intercalating cyanine dye **BO** has been extended with a benzothiazole substituent. The resulting crescent-shape of the molecule is similar to that of the well-known minor groove binder Hoechst 33258. Indeed, comparative studies of **BO** illustrate a considerable change in binding mode induced by this structural modification. Linear and circular dichroism studies indicate that **BEBO** binds in the minor groove to [poly (dA-dT)]$_2$, but that the binding to calf thymus DNA is heterogeneous, although still with a significant contribution of minor groove binding. Similar to other DNA binding asymmetric cyanine dyes, **BEBO** has a large increase in fluorescence intensity upon binding and a relatively large quantum yield when bound. The minor groove binding of **BEBO** to [poly (dA-dT)]$_2$ affords roughly a 180-fold increase in intensity, which is larger than to that of the commonly used minor groove binding probes DAPI and Hoechst 33258.

Introduction

Asymmetric cyanine dyes have achieved much interest due to their excellent nucleic acid staining properties. Upon binding to nucleic acids such dyes usually exhibit a large enhancement in fluorescence intensity[1] and are widely used as fluorescent markers for DNA in various contexts.[2–4] The interaction between double stranded DNA and the asymmetric cyanine dyes **TO** and **YO** (Fig. 1) have been investigated spectroscopically in several studies and were found to bind by intercalation[5–7] in a non-specific fashion.[8] They also bind strongly to single stranded DNA with a large accompanying increase in fluorescence intensity.[9] This makes the dyes less useful in studies where only a signal from double stranded DNA is desirable. There are, however, fluorescent ligands binding in the minor groove instead of by intercalation that bind selectively to double and not to single stranded DNA, for example, DAPI[10] and Hoechst-derivatives.[11] In contrast to most cyanine dyes these ligands have a DNA sequence selectivity, preferably for A/T-rich segments.[12] Furthermore, compared to the intercalating dyes they exert a smaller perturbation of the DNA-duplex upon binding. This is valuable in studies where its critical that the DNA is not stretched out, for example in certain fluorescence microscopy studies.[13,14] Minor groove binders do not, however, exhibit an equally dramatic increase in fluorescence as the asymmetric cyanine dyes upon binding to DNA, who can display more than a thousand-fold increase.[1] For **BO** (Fig. 1) a 400-fold enhancement in fluorescence has been reported,[15] whereas Hoechst and DAPI exhibit a ~95-fold[16] and a ~20-fold[17] increase, respectively. Another advantage of the asymmetric cyanine dyes as labels for DNA is their relatively long absorption maxima, which reduces problems of background absorption from biological material. The absorption maxima of the dyes in figure 1 when bound to DNA varies from roughly 435 nm to 510 nm[6,9,17] compared to around 350 nm for Hoechst 33258 (Hoechst) and DAPI.[17] A dye that combines the features of the minor groove binding ligands and the photophysical properties of the ordinary asymmetric cyanine dyes would thus be of great value for detection and studies of DNA.

As an initial effort towards such a dye we designed the asymmetric cyanine dye **BEBO** (Scheme 1). This dye has the same cyanine chromophore as the intercalating dye **BO** but the structure is extended with a benzothiazole substituent in the 6-position. The positioning of the benzothiazole moiety gives **BEBO** a crescent-shape

*Corresponding author. Tel.: +46-31-772-3072; fax: +46-31-772-3657; e-mail: westman@oc.chalmers.se

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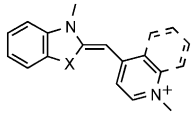
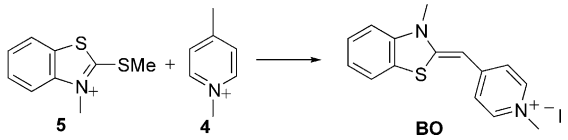


Dye	X	Heterocycle	λ_{max}
BO	S	pyridine	445
TO	S	quinoline	510
PO	O	pyridine	435
YO	O	quinoline	480

Figure 1. Intercalating asymmetric cyanine dyes.



Scheme 2. Reagents and conditions: triethylamine, dichloromethane, rt 14 h.

similar to that of other minor groove binders, for example, Hoechst. The short synthetic route to **BEBO** starting from the commercially available benzothiazole substituted aniline **1** motivated the choice of the benzothiazole group (Scheme 1). In addition, symmetrical cyanine dyes containing two benzothiazole moieties have been suggested to bind in the minor groove either as monomers[18] or as dimers.[19] Herein we describe the synthesis and DNA binding studies of **BEBO** and the analogous dye **BO**.

Results and Discussion

Synthesis

Typically asymmetric cyanine dyes are prepared by condensation of two quaternary heterocyclic salts with a thiomethyl group acting as leaving group on one of the salts. However, the use of an alternative condensation method developed by Deligeorgiev et al.[20] furnished a synthetic route to **BEBO** of only four steps starting from the commercially available 4-substituted aniline **1** (Scheme 1). Thiocyanation of the aniline **1** with potassium thiocyanate and bromine in DMF afforded the 2-aminobenzothiazole **2** in a 40% yield.[21,22] Methylation of **2** by iodomethane and subsequent deprotonation proceeded in a total 77% yield to produce the 2-imino-3-methyl-benzothiazoline **3**. The dye **BEBO** was prepared in 24% by simply melting the benzothiazoline **3** together with the pyridinium salt **4** at 160 °C under vacuum.[20]

To enable comparative DNA binding studies the presumed intercalating dye **BO** (1-methyl-4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]-pyridinium iodide) was synthesised according to the classical method using a modified procedure by Zhou et al. (Scheme 2).[23] The dye was afforded in 46% yield by condensation of the pyridinium salt **4** and the benzothiazolium salt **5** in dichloromethane using triethyl amine as base.

Linear dichroism measurements

To study the effect induced by the benzothiazole substituent in **BEBO** on its interaction with DNA, binding studies of the analogous dye **BO** were also performed as a comparison. Figure 2 shows the flow linear dichroism (LD) spectra of **BEBO** and **BO** with different DNA. LD is defined as the difference in absorption of light polarized parallel and perpendicular to the macroscopic axis of orientation. The LD-spectra of oriented DNA-ligand complexes may be analysed in terms of angles that the electronic transition moments of the ligands make with the DNA-helix axis to provide information about binding geometries.[24] The orientation of the DNA complexes was achieved using a flow Couette cell with outer rotating cylinder. For **BEBO** in presence of [poly(dA-dT)]$_2$ (poly-AT) a clear positive LD is shown providing a strong indication of minor groove binding (Fig. 2). From the reduced LD, obtained through division of the LD by the isotropic absorption, the angle between the long wavelength transition moment of **BEBO** and the DNA-helix was calculated to be 44°. This is very

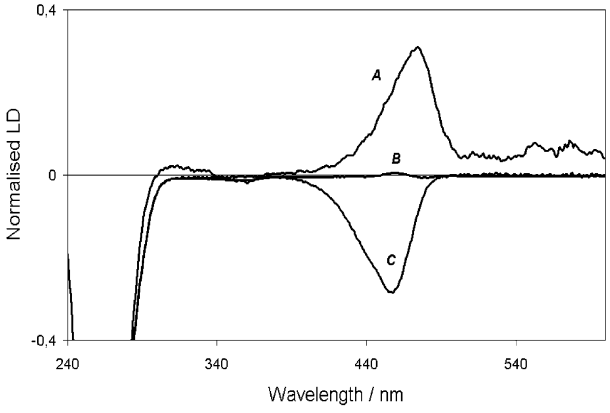
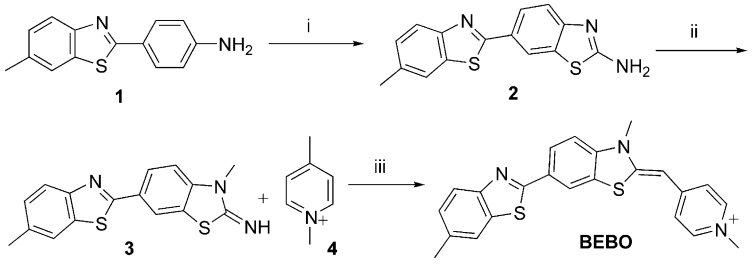


Figure 2. Flow LD spectra of BEBO complexed with: (A) [poly(dA-dT)]$_2$, (B) ctDNA and (C) BO complexed with ctDNA, normalised at the DNA base transition. Binding ratio *R*, dye:bases, were 0.05. [dye] = 11 μM in all spectra.



Scheme 1. Reagents and conditions: (i) Br_2, KSCN, DMF, 3 h; (ii), 1. MeI, DMSO, 17 h, 110 °C, 2. $NaOH_{aq}$, DMSO; (iii), 160 °C, vacuum 1 h.

similar to the angle for known minor groove binders, for example, DAPI.[25] The major transition moment of **BEBO** can be expected to be polarized roughly along the line connecting the pyridine with the closest benzothiazole ring.[26] The weaker positive signal shown for **BEBO** in presence of calf thymus DNA (ctDNA) is possibly due to binding in the minor groove with an angle close to 54°, as suggested in earlier studies of symmetrical cyanine dyes.[13] However, the binding-angle to poly-AT of 44° in addition with CD-titration data (see below) proposes a more complicated binding to ctDNA with a mixture of binding modes resulting in an average low LD signal. Although Hoechst and DAPI have a preference for minor groove binding to AT-rich regions it has been suggested that they bind to GC sequences by a non-classical intercalation process.[27,28] This model seems to be applicable here also, since the reduced LD spectrum of **BEBO** with $[poly(dG\text{-}dC)]_2$ (poly-GC) show a negative value of the same amplitude as for the DNA bases indicating intercalation (supplementary material).

In contrast to the binding of **BEBO**, LD measurements indicate that **BO** binds by intercalation to all three different polynucleotides studied: ctDNA (Fig. 2), poly-AT and poly-GC (Fig. 3). The change in binding mode induced by the benzothiazole extension of the **BO** structure is particularly apparent in the case of poly-AT.

Circular dichroism measurements

The strong induced positive CD for **BEBO** in presence of poly-AT (Fig. 4a) gives further strong support for binding in the minor groove.[29] Figure 4a shows the titration of poly-AT into **BEBO** with binding ratios *R*, defined as the total number of dye molecules per base, varying from 0.025 to 0.1. The larger CD amplitude of **BEBO** at the highest binding ratio is rationalized by a contribution of exciton coupling interactions between closely bound chromophores. This is illustrated by subtracting the *B* spectrum ($R = 0.05$) from the *C* spectrum ($R = 0.1$) in Figure 4a to produce a spectrum typical of exciton coupling (*D*, Fig. 4a).

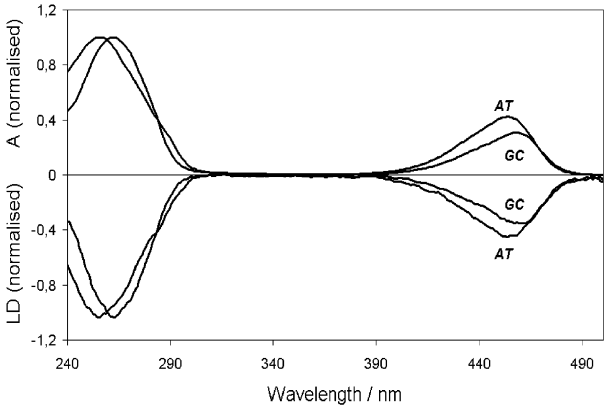


Figure 3. Normalised LD and absorption spectra of BO in presence of: *AT*) $[poly\ (dA\text{-}dT)]_2$, *GC*) $[poly\ (dG\text{-}dC)]_2$. [BO] = 11 μM. $R = 0.025$.

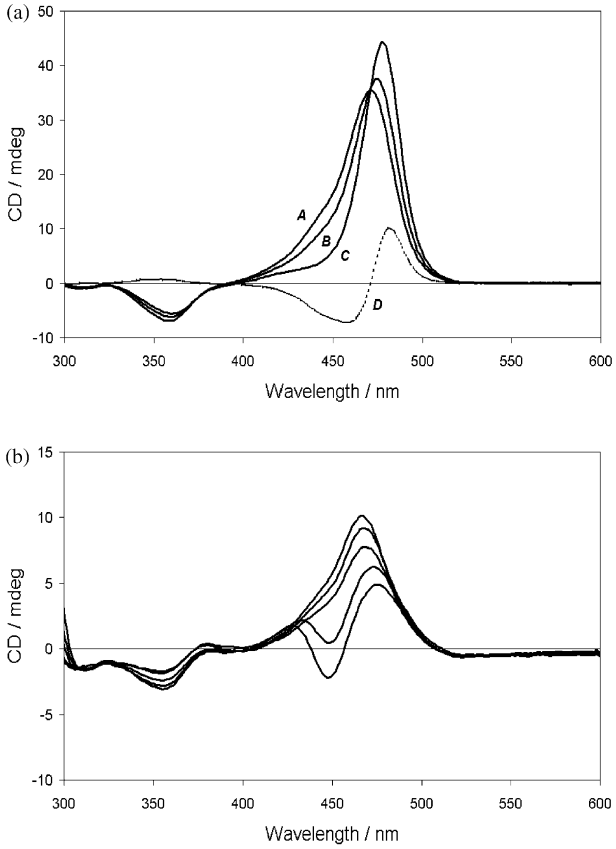


Figure 4. CD spectra of BEBO in presence of (a) $[poly\ (dA\text{-}dT)]_2$, [$R = 0.025$ (A), 0.05 (B), 0.10 (C), (D) = (C) −(B)] and (b) ctDNA (*R* values from bottom to top are 0.1, 0.05, 0.033, 0.025 and 0.0125). [dye] = 11 μM in all spectra.

In presence of ctDNA the induced CD (ICD) is smaller but still, intercalation of the dye would not give rise to this large amplitude. The titration of ctDNA into **BEBO** with binding ratios *R* varying from 0.0125 to 0.10 is shown in Figure 4b. As with the binding of **BEBO** to poly-AT, there is a feature of exciton coupling interactions between closely spaced ligands at higher binding ratios. At lower binding ratio the signal is similar to that of the corresponding poly-AT spectra, albeit with smaller amplitude. There are cases of external stacking giving rise to a large ICD due to exciton coupling between the ligands.[30] However this binding mode can be ruled out here since this coupling also would lead to a splitting of the ICD band into a positive and a negative component. Thus, there must be a significant amount of dye residing in the minor groove.

The binding of **BEBO** to poly-GC give rise to only a very small ICD (Fig. 5a), which supports an intercalative binding mode to GC-regions. This might partly account for the lower CD obtained upon binding to ctDNA. However, one must bear in mind that ctDNA is more complex than just a mixture of alternating GC- and AT-segments. The amplitude of the CD spectra in presence of ctDNA is about one fourth of the poly-AT spectra. If the binding to ctDNA is a mixture of groove binding to AT-regions and intercalation to GC-regions then 75 percent would be bound in an intercalative fashion. This does not hold since the LD should be significantly more

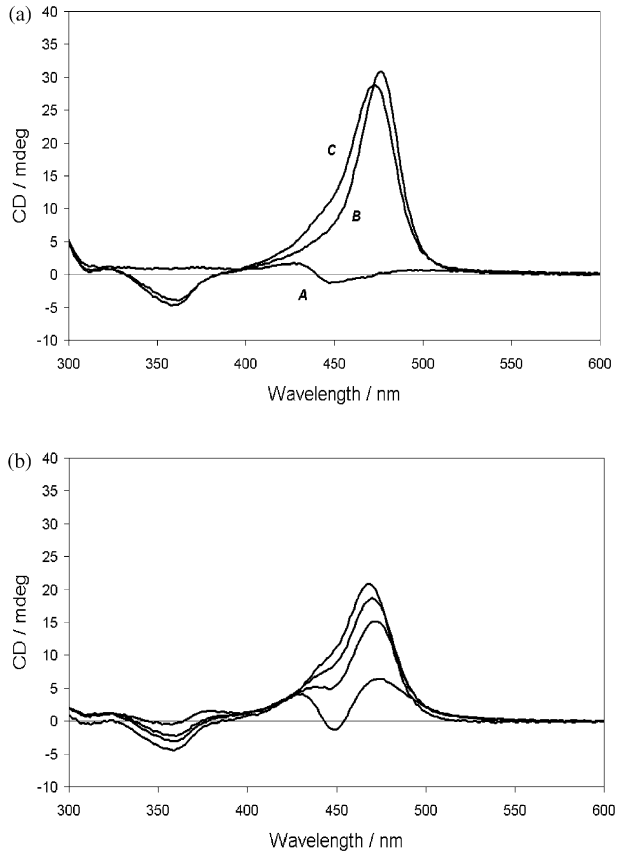


Figure 5. Change in CD after addition of $[poly (dA\text{-}dT)]_2$ into samples of BEBO in presence of (a) $[poly (dG\text{-}dC)]_2$ and (b) ctDNA ($R = 0.05$ in both figures). $[poly (dA\text{-}dT)]_2$ was added to give mixing ratios, dye:AT-bases of: (a) *(B)* 0.1 and *(C)* 0.05, (b) from bottom to top: 0.1, 0.05 and 0.025. [dye] = 11 μM in all spectra.

negative in that case. Hence, a substantial amount of dye must be bound in a non-intercalative fashion to ctDNA at sites affording a lower ICD than when bound to alternating AT.

The CD signal for **BO** in presence of ctDNA was only weakly negative (data not shown) and this further illustrates the different binding mode of **BEBO** compared to **BO**.

Polynucleotide binding preferences

The extensive difference in amplitude of the CD signal for **BEBO** in presence of poly-GC and poly-AT allowed a simple experiment to investigate a possible AT preference. When poly-AT was added to a sample of **BEBO** in presence of poly-GC ($R = 0.05$) the CD signal increased drastically showing a considerable preference for poly-AT (Fig. 5a). These spectra were consistent with the CD spectra of **BEBO** in presence of poly-AT without poly-GC (Fig. 4a) with only slightly lower amplitudes of the signals.

A similar experiment was performed to compare the binding affinities of **BEBO** to poly-AT and ctDNA. Again poly-AT was added to a sample of **BEBO** now in presence of ctDNA. There was an increase in CD signal upon addition of poly-AT but not as large as when the sample initially contained poly-GC (Fig. 5b). Hence, there is still a reasonable amount of dye bound to ctDNA at these ratios showing that there must be other binding sites than alternating AT-regions in ctDNA that attract **BEBO** significantly.

Fluorescence and absorbance measurements

The absorption and fluorescence properties of BEBO with different nucleic acids are summarised in Table 1. In analogy with other asymmetric cyanine dyes, **BEBO** has a large increase in fluorescence upon binding to DNA. The clear minor groove binding of **BEBO** to poly-AT affords a 180-fold enhancement in fluorescence intensity, whereas the increases upon binding to ctDNA and poly-GC are somewhat larger. In buffer solution, the free dye has its emission peak at 542 nm compared to 492 nm for the bound dye. Using ethanol instead of aqueous buffer as solvent the free dye emission was shifted to 492 nm, and the fluorescence intensity was roughly ten times lower. Recently aggregation of **TO** in presence and absence of DNA was studied by absorption and fluorescence spectroscopy and similar manifestations caused by aggregate formation was seen.[31] Thus in buffer solution dimers or higher aggregates with longer emission maximum are probably formed due to the hydrophobic nature of the dye.

The shape of the absorption spectra of free **BEBO** in water further suggests the presence of dimers or aggregates (Fig. 6). Absorption measurements of **BEBO** in different methanol–water mixtures showed a substantial increase and a red shift in absorption with increasing amount of methanol (Fig. 6b). The dye molecules are presumably present as monomers in pure methanol. The absorption spectrum of free **BEBO** in methanol and the spectrum of **BEBO** completely bound to DNA have a very similar shape, which indicates that the dye is bound as monomers at low binding ratios.

Conclusions

In summary, we find that the structural modifications of **BO** have induced a shift in binding mode from intercalation towards minor groove binding. Our results further imply the potential of the benzothiazole group as a minor groove recognition moiety. The dye could be synthesised in four steps only from the commercially available aniline **1**. The binding of **BEBO** to poly-AT is clearly in the minor groove as deduced from the CD- and LD-spectra. Similarly to that of DAPI and Hoechst, the binding of **BEBO** to poly-GC is dominated by intercalation. With the random sequence ctDNA on the other hand, **BEBO** seems to interact heterogeneously. However, intercalation to GC-segments and minor groove binding to AT-regions cannot be the only explanation to the LD- and CD-results obtained with ctDNA. There must be other preferred binding sites in ctDNA for BEBO, which induce a lower CD than poly-AT. The relatively large amplitude of the CD signal show, however, that there is a significant contribution of minor groove binding of **BEBO** to ctDNA. Consistent

Table 1. Fluorescence and absorbance properties of BEBO free in buffer and bound to different DNA[a]

	Abs. peak (nm)	Em. peak (nm)	ϕ_F[c]	F_{bound}/F_{free}[d]
Free BEBO	448	542	0.011	
BEBO-ctDNA[b]	467	492	0.18	245
BEBO-polyAT[b]	467	492	0.118	182
BEBO-polyGC[b]	471	492	0.226	264

[a]Measured at 25 °C in 10 mM sodium phosphate buffer (pH 7.0).
[b]Dye/bases ratio of 1:100.
[c]Fluorescence quantum yields, ϕ_F, were determined relative to fluorescein in 0.1 M NaOH, assuming a ϕ_F of 0.93.
[d]Increase in fluorescence intensity at 492 nm when exciting at 467 nm.

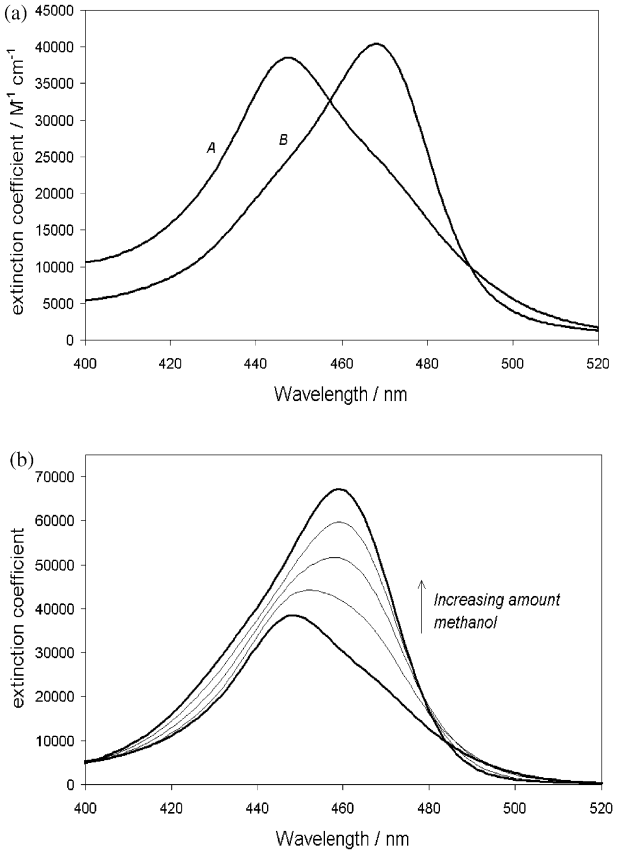


Figure 6. (a): Absorption spectra of BEBO free in buffer (*A*) and bound to calf thymus DNA (*B*) at *R* value of 0.02; (b): absorption spectra of free BEBO in water-methanol solutions with different compositions ranging from 0 to 100% methanol (thickened lines).

with other minor groove binders **BEBO** has a distinct preference for poly-AT compared to poly-GC. The fluorescence increase upon binding to the minor groove of poly-AT is larger than for Hoechst and DAPI. The binding properties of **BEBO**, in particular its strict minor groove binding to poly-AT, give promise for the development of a new class of asymmetric cyanine dyes with a strong preference for minor groove binding and a large increase in fluorescence upon binding. Synthesis and studies of analogous dyes are underway and will be reported in due time.

Experimental

Column chromatography was performed using aluminium oxide (activated, neutral, approx. 150 mesh) deactivated by addition of water to Brockman grade III. Melting points were determined on a Mettler FP82HT hot-stage microscope. ^{1}H (400 MHz) and ^{13}C (100.6 MHz) NMR spectra were recorded at rt using a Varian UNITY-400 NMR spectrometer. Chemical shifts are in ppm, relative to solvent peaks for DMSO (δ 2.50 for ^{1}H and δ_C 39.51 for ^{13}C NMR); *J* values are given in Hz. High resolution mass spectra were recorded using a VG ZabSpec instrument. UV–vis spectra were measured on a Varian Cary4 spectrophotometer. Fluorescence spectra were recorded using a SPEX fluorolog τ2 spectrofluorimeter. The LD and CD spectra were recorded on a JASCO-720 spectropolarimeter. The orientation of the DNA complexes was achieved using a flow Couette cell with outer rotating cylinder. All spectroscopic measurements were performed at 25 °C in 25 mM sodium phosphate buffer (pH 7.0). Aqueous solutions of BEBO and BO were typically obtained from 2 mM stock solutions in DMSO. [Poly (dA-dT)]$_2$ and [poly (dG-dC)]$_2$) were purchased as solutions in buffer from Pharmacia. Calf thymus DNA was purchased from Fluka. Commercial reagents were purchased from Sigma-Aldrich and used without further purification. The pyridinium salt **4** and the benzothiazolium salt **5** were prepared as previously reported.[23]

2-Amino-6-(6-methyl-benzothiazol-2-yl)-benzothiazole (2)

2-(4-Aminophenyl)-6-methyl-benzothiazole **1** (4.0 g, 16.6 mmol) and KSCN (2.6 g, 26.7 mmol) were dissolved in DMF (20 mL) and cooled in an ice-bath. Br_2 (0.9 mL, 17 mmol) in DMF (15 mL) was added dropwise under 3 h. The mixture was stirred for another 20 h. Water was added and the precipitate formed was collected by filtration and dried. The crude product was triturated on the sinter with several portions of boiling dichloromethane to afford **2** as a light green-yellow solid (1.97 g, 40%). Mp 250–251 °C; ^{1}H NMR (DMSO) δ 2.45 (3H, s, Ar–CH_3), 7.34 (1H, d, *J* = 8.4, ArH), 7.50 (1H, d, *J* = 8.4, ArH), 7.89 (1H, d, *J* = 8.4, ArH), 7.91 (1H, s, ArH), 7.99 (1H, d, *J* = 8.4, ArH), 8.51 (1H, s, ArH), 8.56 (2H, s, NH_2); ^{13}C NMR (DMSO): δ 21.10, 116.7, 120.7. 121.8, 122.1, 125.6, 126.9, 128.1, 129.7, 133.3, 134.5, 135.0, 151.7, 165.9, 169.3; HR-FAB-MS *m*/*z*: found: 298.0521 $C_{15}H_{12}N_3S_2$ ($M+H^+$): requires M, 298.0473.

2-Amino-3-methyl-6-(6-methyl-benzothiazol-2-yl)-benzothiazolium iodide. The 2-aminobenzothiazole **2** (0.3 g, 1.0 mmol) was dissolved in DMSO (2 mL). Methyl iodide (0.25 mL, 2.0 mmol) was added and the mixture was stirred at 110 °C for 17 h. The mixture was cooled and poured into water. The precipitate formed was collected by filtration and washed with water to give the product as a yellow solid (0.38 g, 86%). Mp 267–269 °C; ^{1}H NMR (DMSO): δ 2.47 (3H, s, Ar–CH_3), 3.74 (3H, s, N–CH_3), 7.38 (1H, d, *J* = 8.4, ArH), 7.79 (1H, d, *J* = 8.4, ArH), 7.93 (1H, d, *J* = 8.4, ArH), 7.95 (1H, s, ArH), 8.22 (1H, d, *J* = 8.4, ArH), 8.75 (1H, s, ArH), 10.19 (2H, s, NH_2); ^{13}C NMR (DMSO): δ 21.14, 32.39, 113.9, 122.0,

122.2, 122.4, 122.7, 126.6, 128.3, 129.8, 134.7, 135.6, 140.9, 151.6, 164.7, 168.9; HR-FAB-MS *m/z*: found: 312.0638 $C_{16}H_{14}N_3S_2$ (M^+): requires M, 312.0629.

2-imino-3-methyl-6-(6-methyl-benzothiazol-2-yl)-benzothiazoline (3). 2-Amino-3-methyl-6-(6-methyl-benzothiazol-2-yl)-benzothiazolium iodide (0.3 g, 0.68 mmol) was taken up in DMSO (10 mL). Water was added (20 mL) and the mixture was basified to pH 10 with aqueous NaOH (20%). The precipitate was collected by filtration and washed with water to produce **3** as a light yellow solid (0.19 g, 89%). Mp 146–148 °C; ^{1}H NMR (DMSO): 2.45 (3H, s, Ar–CH_3), 3.38 (3H, s, N–CH_3), 7.16 (1H, d, J=8.4, ArH), 7.33 (1H, d, J=8.4, ArH), 7.87 (1H, s, ArH), 7.90 (1H, s; ArH), 7.93 (1H, d, J=8.4, ArH), 8.16 (1H, s, ArH), 8.55 (1H, s, NH); ^{13}C NMR analysis was not possible due to poor solubility of **4** in available deuterated solvents; HR-FAB-MS *m/z*: found: 312.0619 $C_{16}H_{14}N_3S_2$ ($M+H^+$): requires M, 312.0629

4-[(3-Methyl-6-(6-methyl-benzothiazol-2-yl)-2,3,-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium iodide (BEBO). The benzothiazoline **3** (0.1 g, 0.32 mmol) and 1,4-dimethyl-pyridinium tosylate **4** was melted together at 160 °C under vacuum for 1 h. DMSO (5 mL) was added and the mixture was heated at reflux for 30 min. The mixture was added to aqueous KI (30%) and the precipitate formed was collected by filtration. The solid was purified by flash chromatography on neutral Al_2O_3 with methanol–dichloromethane (2:98) to give **BEBO** (0.04 g, 24%). Mp 280–281 °C; ^{1}H NMR (DMSO): δ 2.47 (3H, s, Ar–CH_3), 3.76 (3H, s, N–CH_3), 4.02 (3H, s, N–CH_3), 6.34 (1H, s, =CH–), 7.38 (1H, d, J=8.4, ArH), 7.47 (1H, d, J=6.8, PyH), 7.70 (1H, d, J=8.4, ArH) 7.93 (1H, d, J=8.4, ArH), 7.95 (1H, s, ArH), 8.18 (1H, d, J=8.4, ArH), 8.39 (1H, d, J=6.8, PyH), 8.65 (1H, s, ArH); ^{13}C NMR (DMSO): 21.13, 32.99, 45.11, 90.66, 112.0, 118.8, 120.9, 121.8, 122.2, 124.6, 126.7, 127.9, 128.1, 134.5, 135.2, 142.4, 142.5, 150.1, 151.6, 156.4, 164.9; HR-FAB-MS *m/z*: found: 402.1145 $C_{23}H_{20}N_3S_2$ (M^+): requires M, 402.1105.

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