Groove-binding unsymmetrical cyanine dyes for staining of DNA: syntheses and characterization of the DNA-binding

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Received July 7, 2003; Revised August 20, 2003; Accepted September 9, 2003

ABSTRACT

Two new crescent-shaped unsymmetrical cyanine dyes have been synthesised and their interactions with DNA have been investigated by different spectroscopic methods. These dyes are analogues to a minor groove binding unsymmetrical cyanine dye, BEBO, recently reported by us. In this dye, the structure of the known intercalating cyanine dye BO was extended with a benzothiazole substituent. To investigate how the identity of the extending heterocycle affects the binding to DNA, the new dyes BETO and BOXTO have a benzothiazole group and a benzoxazole moiety, respectively. Whereas BEBO showed a heterogeneous binding to calf thymus DNA, linear and circular dichroism studies of BOXTO indicate a high preference for minor groove binding. BETO also binds in the minor groove to mixed sequence DNA but has a contribution of non-ordered and non-emissive species present. A non-intercalative binding mode of the new dyes, as well as for BEBO, is further supported by electrophoresis unwinding assays. These dyes, having comparable spectral properties as the intercalating cyanine dyes, but bind in the minor groove instead, might be useful complements for staining of DNA. In particular, the benzoxazole substituted dye BOXTO has attractive fluorescence properties with a quantum yield of 0.52 when bound to mixed sequence DNA and a 300-fold increase in fluorescence intensity upon binding.

INTRODUCTION

Over the past decades, there has been extensive progress on the design of small molecules recognizing the minor groove of B-DNA (1–4), and it is now apparent that the minor groove is an important carrier of DNA sequence information. Understanding the features that contribute to recognition of DNA by small ligands is crucial for the development of drugs

targeted at DNA, such as antitumor agents for example (5). Two thoroughly studied minor groove binders (MGBs) are Hoechst 33258 (Hoechst) (6–8) and DAPI (8,9), which bind preferentially at AT-rich regions of B-DNA (10,11). Upon binding to DNA, these ligands exhibit an increase in fluorescence intensity (12), which has enabled them widespread use as fluorescent markers for DNA in various contexts (9,13,14). Although the increase in fluorescence by DAPI and Hoechst upon binding to DNA is relatively modest, about 20- and 30-fold, respectively (12,15), an attractive feature of MGBs is their selectivity for double-stranded compared to single-stranded DNA (15).

By comparison, intercalating unsymmetrical cyanine dyes such as thiazole orange (TO) and oxazole yellow (YO) (Fig. 1) can display more than a 1000-fold increase in fluorescence upon binding to DNA (16–19). Due to their superior DNA-staining properties, these cyanines are currently used in various biotechnical applications (20–25). However, in contrast to MGBs, the unsymmetrical cyanines are almost devoid of DNA sequence selectivity (26) and fluoresce strongly both to single- and double-stranded DNA (27,28). The insertion of the dye molecules between the base pairs leads to an extension of the DNA helix (29,30), a potential disadvantage in for example studies of hydrodynamic and flexibility properties of DNA in solution (31), where the polymer length is the crucial variable. Binding in the minor groove is not expected to significantly lengthen the DNA-helix. Indeed, in fluorescence microscopy studies, DAPI has been reported to exhibit a smaller effect on the DNA conformation than the intercalator ethidium bromide (30), as well as exhibiting a negligible effect on the folding transition of isolated giant DNA, which was markedly prevented by YOYO (32), although the contrast of the fluorescence images of the DNA was less satisfactory.

Thus, dyes combining the favourable spectral properties of the intercalating cyanine dyes, but binding in the minor groove, would be useful complements for staining of DNA. Apart from having larger enhancements in fluorescence than available minor groove binding stains (Hoechst and DAPI), such dyes could also be excited at longer wavelengths. Although Hoechst and DAPI can be independently visualized in the presence of fluorophores emitting at longer wavelengths, they both absorb in the near-UV region where background from scattering becomes extensive.

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Figure 1. The unsymmetrical cyanine dyes BEBO, BETO and BOXTO.

Recently, we reported on an unsymmetrical cyanine dye, BEBO (Fig. 1) that exhibits minor groove binding properties (33). In this dye, the structure of the known intercalating dye BO was extended with a benzothiazole substituent. The resulting crescent-shape of the molecule is similar to that of Hoechst. Indeed, linear and circular dichroism studies showed that BEBO binds in the minor groove of poly(dA-dT)₂. With the mixed sequence calf thymus DNA (ctDNA) on the other hand, BEBO appears to interact heterogeneously, although with a significant contribution of minor groove binding. Similarly to that of DAPI and Hoechst, the binding of BEBO to poly(dG-dC)₂ is dominated by intercalation, and BEBO has a distinct preference for poly(dA-dT)₂ compared to poly(dG-dC)₂. The binding of BEBO to the different DNA studied was accompanied by roughly a 200- to 300-fold enhancement in fluorescence intensity around the emission maximum (33,34). In another study, BEBO has been shown to be a good complement to SYBR Green I as a non-specific probe in real-time PCR (34).

The heterogeneous binding of BEBO to mixed sequence DNA prompted us to search for derivatives with a higher preference for the minor groove, and possibly with improved fluorescence enhancement upon DNA binding. The introduction of an extending benzothiazole group in BEBO clearly induced a change in binding mode from intercalation towards minor groove binding. To investigate how the nature of the extending heterocycle affects the binding to DNA, we set out to prepare dyes with benzoxazole (BOXTO) and benzothiazole extensions (BETO) (Fig. 1). In contrast to the previous synthetic route towards BEBO, a new strategy was developed that facilitates the preparation of dyes with different extending heterocycles. As judged from the linear and circular dichroism studies, the benzoxazole derivative BOXTO exhibited straightforward minor groove binding both to poly(dA-dT)₂ and calf thymus DNA (ctDNA), whereas the benzothiazole derivative BETO showed a more heterogeneous binding to the latter DNA, also with a greater tendency for aggregation. Herein we present the synthesis and DNA-binding studies of these new unsymmetrical cyanine dyes. In an accompanying paper, we present a study of the dissociation of these dyes from DNA under conditions typical for biotechnical application (35).

MATERIALS AND METHODS

Spectroscopic measurements

UV-vis spectra were measured on a Varian Cary4 spectrophotometer. Fluorescence spectra were recorded using a

SPEX fluorolog τ_2 spectrofluorimeter. The flow-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 5 mM sodium phosphate buffer (pH 7.0). Aqueous solutions of the dyes used were typically obtained from 1–3 mM stock solutions in DMSO. The DNA concentration per nucleotide was determined by absorption spectroscopy using extinction coefficients of 6600 M⁻¹ cm⁻¹ at 260 nm for both ctDNA and poly(dA-dT)₂, and 8400 M⁻¹ cm⁻¹ for poly(dG-dC)₂. BOXTO and BETO concentrations were estimated using extinction coefficients of 37 800 and 38 000 M⁻¹ cm⁻¹, respectively. Poly(dA-dT)₂ and poly(dG-dC)₂ were purchased as solutions in buffer from Pharmacia. ctDNA was purchased from Fluka.

Unwinding experiments

Unwinding of supercoiled doubled-stranded circular DNA ϕ X174 (RFI super coiled and RFII relaxed form), 5386 bp (MBI Fermentas), was monitored during constant field gel electrophoresis (3 V cm⁻¹) in agarose gels. Agarose was obtained as a dry powder from Sigma-Aldrich. Equal amounts of supercoiled and relaxed form (nicked circle) DNA ϕ X174 were used in each sample (in total, 0.25 μ g DNA per sample). Mole fractions of dye/DNA base between 0.01 and 0.16 were studied. Scanning of the gels was performed by exciting the samples at 488 or 514 nm depending on the absorption maximum of the dye and using a longpass emission filter with 530 or 570 nm cut-off, respectively. The difference in migration velocities of the supercoiled and the relaxed forms of DNA at different binding ratios of the dyes was evaluated using ImageQuant 5.0.

RESULTS AND DISCUSSION

Synthesis

The syntheses of the dyes are presented in Figure 2 and the experimental details are given in the Supplementary Material. The recently reported synthetic strategy of BEBO has a limitation in that all dyes obtained would have the benzothiazole moiety as the extending heterocycle. Facilitating the preparation of dyes with different extending heterocycles, to provide new analogues of BEBO, we here use a Stille cross-coupling strategy. The 6-halo-2-methyl-benzothiazoles **5a** and **5b** were prepared in three and four steps, respectively, starting from the appropriate anilines (36–38). Attempted Stille couplings of 6-bromo-2-methylbenzothiazole **5a** with both of the organostannanes **6a** and **6b** gave no trace of coupled product. However, coupling reactions with the iodo analogue **5b** gave the heteroaryl-benzothiazoles **7a** and **7b** in almost quantitative yields, consistent with the generally higher reactivity of aryl-iodides in palladium catalysed cross-couplings (39).

The dyes BOXTO and BETO were achieved by condensation of the benzothiazolium salts **8** and the quinolinium salt **9** in dichloromethane using triethyl amine as base (Fig. 2). Unfortunately, attempts to react the benzothiazolium salts with N-methyl-4-thiomethyl-pyridinium tosylate to obtain the pyridine dye derivatives were unsuccessful. Under basic conditions, the benzothiazolium salt can react with itself

Figure 2. Reagents and conditions: (i) KBr, $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$, AcOH, rt, 22 h; (ii) Ac_2O , pyridine, 2 h, 100°C ; (iii) phosphorus pentasulfide, benzene, reflux, 2 h; (iv) NaOMe, NMP, 150°C , 2 h; (v) $\text{Pd}(\text{dba})_3$ [0.1 equiv. Pd(0)], PPh_3 (0.4 equiv.), CuI (0.4 equiv.), DMF, 60°C , 6 h; (vi) MeOTs, 90°C , 5 h; (vii) NEt_3 , CH_2Cl_2 , rt, 48 h.

Table 1. Fluorescence and absorbance properties^a of BETO and BOXTO in comparison with known MGBs and the intercalating cyanine dye TO

	$\lambda_{\text{abs.max}}$ (nm)	$\lambda_{\text{ems.max}}$ (nm)	Φ_F^c	$\Phi_{\text{bound}}/\Phi_{\text{free}}$	$F_{\text{bound}}/F_{\text{free}}^d$	Reference
Free BETO	487	590	0.015			This work
BETO-ctDNA ^b	516	561	0.21	14	130	This work
Free BOXTO	482	588	0.011			This work
BOXTO-ctDNA ^b	515	552	0.52	50	260	This work
Free TO	501	—	0.0002			(27)
TO-ctDNA	508	525	0.11	550		(27)
Free BEBO	448	542	0.011			(33)
BEBO-ctDNA	467	492	0.18	16	245	(33)
DAPI-ctDNA	349	456	0.34	18		(15)
Hoechst-ctDNA	356	466	0.42	28		(15)

^aMeasured at 25°C in phosphate buffer (5 mM, pH 7.0).

^bDye/bases ratio 1:100.

^cFluorescence quantum yields, Φ_F , were determined relative to fluorescein in 0.1 M NaOH, assuming a Φ_F of 0.93.

^d $F_{\text{bound}}/F_{\text{free}}$ is the increase in fluorescence intensity upon binding to DNA of BETO and BOXTO at 526 nm and of BEBO at 492 nm.

producing a red-purple by-product only. Thus, even though the 4-thiomethyl-pyridinium salt has a better leaving group than the quinolinium salt **9**, the latter is more reactive in these condensations.

Absorbance measurements

The absorption and fluorescence properties of BETO and BOXTO in the absence and presence of ctDNA are summarised in Table 1. The corresponding properties of TO, Hoechst and DAPI are also included in Table 1 for comparison. Whereas Hoechst and DAPI absorb in the near-UV region, the absorbance maxima of both BOXTO and BETO bound to ctDNA are at around 515 nm.

The absorption titration of ctDNA into samples of BOXTO (top) and BETO (bottom) are shown in Figure 3, respectively (throughout the entire paper, spectra of BOXTO will appear in the top panel of figures and spectra of BETO in the bottom panels), with mixing ratios, R , defined as the total number of dye molecules added per base, ranging from 0.267 to 0.0067. Going from high to low mixing ratios, there is a red-shift and an increase in the absorbance of both dyes. The absorption spectrum of free BOXTO in acetonitrile (data not shown), where the dye is most likely to be present as monomers, and the spectrum of BOXTO at the lower mixing ratios have a very similar shape, indicating a monomeric binding mode. It is known that cyanine dyes form molecular aggregates readily, particularly in water (40,41). Thus in buffer solution, dimers or higher aggregates are probably formed due to the

hydrophobic nature of the dyes. The peaks at around 490 nm seen for free dyes in buffer and at high mixing ratios are presumably due to the presence of either free or bound dimers. For BETO, this shoulder is more pronounced even at low mixing ratios, indicating a higher abundance of dimer interaction of BETO with ctDNA, which is further supported by CD measurements (*vide infra*).

Fluorescence measurements

In analogy with BEBO, the fluorescence enhancements displayed by BETO and BOXTO upon binding to DNA are large (Table 1). The high quantum yield of these new cyanines bound to DNA combined with their absorption at long wavelengths may render them useful in biological applications, for example in fluorescence microscopy studies. In particular, BOXTO has a large quantum yield of 0.5, comparable to the homodimeric cyanine dyes YOYO and TOTO (18). In comparison with the parent monovalent intercalating dye TO the quantum yield of bound BOXTO is about five times higher. However, due to the insignificant fluorescence of free TO in solution, the quantum yield enhancement upon binding of TO is still larger than that of BOXTO.

Fluorescence titrations of ctDNA into BOXTO and BETO (Supplementary Material fig. 2) show that at the lowest mixing ratio (dye/DNA-bases 1:150), BOXTO exhibits a 340-fold increase in fluorescence intensity, whereas BETO shows only a 150-fold increase. The normalized spectra of the new dyes at

Figure 3. Absorbance titrations of ctDNA into samples of BOXTO (top) and BETO (bottom). Mixing ratios, dye/bases, from bottom to top are 0.267, 0.133, 0.067, 0.033, 0.0167 and 0.0067. Spectrum of free dye in water is indicated.

different mixing ratios are almost superimposable, indicating one dominant fluorescent species (Supplementary Material fig. 2). Thus, the bound dye molecules giving rise to the emission most probably bind as monomers with a similar binding mode throughout the DNA-helix.

Interestingly, BOXTO, BETO and BEBO (33) all display a blue-shift in emission maxima upon binding to DNA (Table 1). This blue-shift can be an advantage since the fluorescence intensity for the free dye at wavelengths near the emission maxima for the bound dye is low, resulting in a greater enhancement in fluorescence intensity when measured at shorter wavelengths compared to the total increase in quantum yield.

The normalized excitation and absorbance spectra for BOXTO and BETO in the presence of ctDNA are shown in Figure 4. The absorbance and excitation spectra of BOXTO are completely superimposed, indicating that all dye molecules are emissive and bound in a similar fashion. In the case of BETO, the absorbance spectrum has a peak at around 490 nm, which clearly does not furnish any fluorescence (Fig. 4, bottom spectra). This is either due to free dye present or possibly to a dimeric binding mode with low fluorescence caused by intermolecular quenching. The latter is in agreement with results obtained by CD (*vide infra*), where a contribution of exciton coupling is also seen around 490 nm.

Flow linear dichroism

Linear dichroism (LD) is the difference in absorption of light polarized perpendicular and parallel to an orientation axis. DNA of sufficient length can become oriented by introducing a flow gradient, and thus, molecules bound to DNA in a

Figure 4. Normalized excitation and absorbance spectra of BOXTO (top) and BETO (bottom) in the presence of ctDNA at a mixing ratio, dye/bases, of 0.033.

specific way will also be oriented. The orientation of the DNA in these studies was achieved using a Couette flow cell with outer rotating cylinder. The LD spectra may be analysed in terms of angles that the electronic transition moments of the dyes make with the DNA-helix axis to provide information about binding geometries (42). A positive LD signal indicates minor groove binding whereas a strong negative signal designates an intercalative binding mode. By dividing the LD spectrum with the absorption spectrum of the randomly oriented sample (isotropic absorption) the reduced LD (LD^r) is obtained. From this value, the angle between the transition moments of the ligands and the DNA-helix axis can be calculated using equation 1:

$$LD^r = S \cdot O = 3/2 \cdot S \cdot (3\cos^2\alpha - 1)$$

The magnitude of the LD^r value depends on two factors, S and O , the orientation and the optical factor, respectively. S reflects the degree of orientation in the system and O is related to the angle, α , between the transition moment direction and the orientation axis. The orientation factor S for the helix axis with respect to the flow axis was calculated from LD^r at 260 nm assuming an average $\alpha = 86^\circ$ for the DNA bases (42).

MGBs such as DAPI and Hoechst bind with an angle of around 45° compared to the DNA axis (10) while intercalators bind with an angle close to 90° (43).

In Figure 5, the normalized flow LD spectra of BOXTO and BETO are shown together with their normalized absorbance spectra (all spectra normalized at the DNA-base transitions). In contrast to LD results for BEBO with the mixed sequence ctDNA, there is clearly a positive signal for both dyes,

Figure 5. Normalized flow-LD and absorbance spectra of BOXTO (top) and BETO (bottom) in the presence of ctDNA at a mixing ratio, dye/bases, of 0.033. Normalized at the DNA-base transition (260 nm). The LD signals are multiplied five times above 400 nm. [dye] = 13.5 μ M in all spectra.

providing an indication of minor groove binding. At this mixing ratio (dye/bases 1:30), the LD signal for BOXTO in the visible region is very similar to its isotropic absorption spectrum. From the reduced LD the angle between the long wavelength transition moment of BOXTO and the DNA helix was calculated using equation 1 to be between 40° and 43°, depending on the wavelength. This is in fair agreement with the angle for known MGBs (45°) (10).

For BETO in the presence of ctDNA (dye/bases 1:30) (Fig. 5, bottom spectra), the absorption spectrum has a second peak at around 485 nm, which is not present in the LD spectrum. This can be rationalized by the presence of dye molecules that are free in solution or bound in a non-oriented fashion to DNA, thus giving rise to a low average LD signal. It could also be due to a mixture of binding modes with intercalation to GC-sequences as proposed for other MGBs (44,45). However, the similar shape of the LD signal for BETO and BOXTO suggests that the LD obtained for BETO originates from dye molecules in the minor groove combined with the presence of randomly oriented dye rather than from a mixture of binding modes. Using equation 1, the angle at the maximum value of the LD spectra was calculated to be 45°.

Flow-LD measurements of BOXTO and BETO in the presence of poly(dA-dT)₂ were also performed. In both cases, the LD signal is positive and has a similar shape to that of their isotropic absorption spectra, providing a strong indication of minor groove binding (Supplementary Material fig. 3). For both dyes, the LD^r is fairly constant (data not shown) for the cyanine transition and the angles were calculated to be around 45° for both BOXTO and BETO, respectively. As in the case of BEBO, the minor groove binding of BETO is more pronounced to poly(dA-dT)₂ than to that of ctDNA. BOXTO

on the other hand seems to interact by groove binding to both mixed sequence ctDNA and poly(dA-dT)₂.

Circular dichroism

Upon binding of achiral molecules to the chiral nucleic acid helix structure, they acquire an induced CD (ICD), which is characteristic of their interaction with DNA. Compared to the intercalation site between the base pairs, the minor groove of DNA provides a more chiral environment affording a larger ICD of MGBs. In addition, non-rigid ligands binding in the minor groove, such as DAPI, obtain a CD signal induced by a chiral deformation of the molecule when interacting with the DNA helix.

Upon interaction with ctDNA, BOXTO and BETO acquire a large ICD, further supporting minor groove binding (46). The titrations of ctDNA into BOXTO and BETO, with mixing ratios varying from 0.0133 to 0.133, are shown in Figure 6. The stronger preference for minor groove binding by BOXTO is illustrated by roughly a twice as large ICD of BOXTO than that of BETO. The spectrum of BETO more clearly exhibits a small contribution of exciton coupling interactions between closely bound chromophores, with the cross-over of the CD-couplet at around 490 nm. As the DNA concentration increases this exciton coupling weakens as the chromophores likely spread out along the DNA giving weaker electronic coupling.

Figure 7 shows the CD titrations of poly(dA-dT)₂ into BOXTO and BETO with mixing ratios *R* varying from 0.033 to 0.133. Interestingly, the amplitude of the induced CD of BOXTO is almost the same as for the BOXTO-ctDNA complex at the same mixing ratio and dye concentration. This further illustrates that, in contrast to BETO, BOXTO has a similar preference for the minor groove of mixed sequence DNA as to that of poly(dA-dT)₂. Presumably, this reflects a larger preference for alternating AT-sequences of BOXTO than for BETO. The BETO-poly(dA-dT)₂ complexes give rise to a similarly large ICD, indeed about twice the value as in the case of ctDNA. In this context, BETO behaves similarly to BEBO, with a more pronounced binding in the minor groove of poly(dA-dT)₂ than in ctDNA.

To investigate a possible preference of the dyes for alternating AT- compared to GC-sequences and ctDNA, titrations of poly(dA-dT)₂ into samples initially containing the dyes and either ctDNA (Fig. 8) or poly(dG-dC)₂ (Fig. 9) were performed. The data in Figure 9 show that, consistent with other MGBs, both dyes have a considerable preference for poly(dA-dT)₂ compared to poly(dG-dC)₂. On the other hand, the titration of poly(dA-dT)₂ into the sample of BOXTO and ctDNA looks quite similar to that of the titration with ctDNA only (Fig. 6), again illustrating the strong preference of the minor groove of mixed sequence DNA by BOXTO.

When titrating poly(dA-dT)₂ into samples of BETO and ctDNA (Fig. 8, bottom spectra), the behaviour is more complex than with BOXTO, and the results further support the similarities between BETO and BEBO. There is an increase in CD signal upon addition of poly(dA-dT)₂ but not as large as when the sample initially contained poly(dG-dC)₂ (Fig. 9). 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 BETO significantly. Furthermore, a high abundance of free

Figure 6. CD spectra of BOXTO (top) and BETO (bottom) in the presence of ctDNA. Mixing ratios, dye/bases, from bottom to top are: 0.133, 0.088, 0.044, 0.029, 0.023, 0.0133. [dye] = 13.5 μ M in all spectra.

Figure 7. CD spectra of BOXTO (top) and BETO (bottom) in the presence of poly(dA-dT)₂. Mixing ratios, dye/bases, from bottom to top are: 0.133, 0.067, 0.033. [dye] = 13.5 μ M in all spectra.

dye can be ruled out, since in that case the ICD would increase more distinctively upon addition of poly(dA-dT)₂. The contribution of exciton coupling is also seen here even at a ratio of dye/AT-bases of 1:30. This suggests a possible preference for a dimeric binding mode to ctDNA. Studies of the dimerization of symmetrical cyanine dyes in the presence

Figure 8. Change in CD after addition of poly(dA-dT)₂ into samples of BOXTO (top) and BETO (bottom) in the presence of ctDNA (A) at a mixing ratio of 0.067. poly(dA-dT)₂ was added to give mixing ratios, dye/AT-bases/ctDNA-bases, of 1:7.5:15, 1:15:15, 1:30:15 (from bottom to top). [dye] = 13.5 μ M in all spectra.

Figure 9. Change in CD after addition of poly(dA-dT)₂ into samples of BOXTO (top) and BETO (bottom) in the presence of poly(dG-dC)₂ (A) at a mixing ratio of 0.067. poly(dA-dT)₂ was added to give ratios, dye/AT-bases/GC-bases, of (B) 1:7.5:15 and (C) 1:15:15. [dye] = 10 μ M in all spectra.

of γ -cyclodextrin showed that benzoxazolium-containing dyes such as 3,3'-diethyloxadiazocyanine [DiOC₂(5)] require more than a 10-fold higher concentration to dimerize

compared to benzothiazole analogues such as 3,3'-diethylthiadicarbocyanine [DiSC₂(5)] (47,48). To this end, Armitage and co-workers found DiSC₂(5) to bind in the minor groove of DNA as dimers forming helical aggregates on the DNA template (49). The same group also found that DiOC₂(5) was much less prone to dimerize in the presence of duplex DNA than DiSC₂(5) (50). The weaker dimerization of the benzoxazolium dye was argued to be due to the lower hydrophobicity and polarizability of the benzoxazole moiety relative to the benzothiazole. This might imply that BOXTO, having a benzoxazole group, is less susceptible to dimerization and interacts with the DNA mainly as monomers, whereas the driving force for dimerization of BETO upon interaction with DNA is higher.

Electrophoresis unwinding assays

Unwinding of negative supercoiled DNA can be used to monitor binding modes of ligands to DNA. Intercalators are known to unwind the DNA significantly, whereas groove binders are generally considered not to exhibit unwinding (51,52). For the known intercalator YO-PRO, unwinding is observed as a distinct minimum in the ratio of the velocity of the supercoiled and relaxed circular forms of a given plasmid (Fig. 10), where the relaxed form acts as a control for all other effects on DNA velocity due to dye-binding, such as altered electrokinetic charge and change in contour length of the DNA. Figure 10 also shows that in contrast, BEBO, BETO and BOXTO do not exhibit a minimum, but instead have a velocity ratio independent of the mixing ratio. Since all three dyes have dissociation half-times comparable to that of YO-PRO (35), in the order of 100 min or more, it can be concluded that the lack of unwinding is not due to release of the dye. In all three cases, intercalation can therefore be ruled out as a significant binding mode in mixed-sequence DNA.

CONCLUSIONS

The synthesis of BOXTO and BETO proceeded satisfactorily, especially the Stille cross-couplings affording the hetero-arylbenzothiazoles **7** in very good yields. Flow-LD measurements of the new dyes indicated that they both bind in the minor groove of the mixed sequence ctDNA. A non-intercalating binding mode is also supported by CD measurements and electrophoresis unwinding assays. However, BOXTO has a larger preference for the minor groove of DNA than BETO, which is rather curious, considering the very small difference in structure. LD and fluorescence results indicate a considerably higher abundance of non-emissive species of the dye in the samples of BETO and ctDNA than in the BOXTO samples at the same dye/DNA-bases ratio. These are possibly randomly oriented dimers, since there is a contribution of an exciton coupled CD-signal and they furnish no LD. This could alternatively be due to free dye present but this possibility is ruled out by the competition experiments. If free dye was the cause, the ICD upon addition of poly(dA-dT)₂ into samples of BETO-ctDNA would reach a similar amplitude as that of BETO-poly(dA-dT)₂ complexes at low mixing ratios. As with other MGBs, the new dyes have a preference for AT- over GC-sequences. BETO has a more pronounced binding in the minor groove to poly(dA-dT)₂ compared to

Figure 10. The ratio of the velocity of supercoiled (RFI) and relaxed form (RFII) of ϕ X174 DNA during constant field gel electrophoresis (3 V cm⁻¹, 2 h) as a function of different mixing ratios of the dyes (dye/bases). BEBO filled square, BETO filled circle, BOXTO open diamond and YO-PRO filled star (the lines are guides for the eye).

binding to ctDNA, whereas BOXTO interacts very similarly with mixed-sequence DNA to that of poly(dA-dT)₂.

The increase in fluorescence quantum yield upon binding to DNA is relatively large for both dyes: 14-fold for BETO and 50-fold for BOXTO (at a dye/bases ratio of 1:100). Due to a blue-shift in emission maxima upon binding to DNA, the increases in fluorescence intensity when measured at 530 nm are greater than the quantum yield enhancements: 260-fold for BOXTO and 130-fold for BETO. Both dyes have their absorbance maxima over 500 nm when bound to DNA, which reduces problems of absorbance scattering and background emission from biological molecules. These results show that, in particular, BOXTO has good potential as a fluorescent marker binding in the minor groove of mixed sequence DNA, especially when considering the large quantum yield of BOXTO (0.52) when bound to ctDNA.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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