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Syntheses and DNA-binding studies of a series of unsymmetrical cyanine dyes: structural influence on the degree of minor groove binding to natural DNA

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Abstract—Twelve crescent-shaped unsymmetrical dyes have been synthesized and their interactions with DNA have been investigated by spectroscopic methods. A new facile synthetic route to this type of cyanine dyes has been developed, involving the preparation of 6-substituted 2-thiomethyl-benzothiazoles in good yields. The new dyes are analogues to the 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 6-methylbenzothiazole substituent. Herein we further investigate the role of the extending benzazole heterocycle, as well as of the pyridine or quinoline moiety of the cyanine chromophore, for the binding mode of these crescent-shaped dyes to calf thymus DNA. Flow LD and CD studies of the 12 dyes show that the extent of minor groove binding to mixed sequence DNA varies significantly between the dyes. We find that hydrophobicity and size are the crucial parameters for recognition of the minor groove. The relatively high fluorescence quantum yield of many of these cyanines bound to DNA, combined with their absorption at long wavelengths, may render them useful in biological applications. In particular, two of the benzoxazole containing dyes BOXTO and 2-BOXTO show a high degree of minor groove binding and quantum yields of 0.52 and 0.32, respectively, when bound to DNA.

1. Introduction

Dyes that bind to DNA have received considerable attention due to their potential as anti-tumor drugs and as tools to study and visualize DNA.[1] Intercalating unsymmetrical cyanine dyes, for example, Thiazole Orange (TO) (Fig. 1) and its homodimeric counterpart TOTO, are a widely used class of compounds of the latter category.[2] Upon binding to nucleic acids these dyes exhibits a dramatic enhancement in fluorescence intensity. The increase in fluorescence is believed to arise when the rotation or torsion about the methine bridge between the two heterocyclic moieties is restricted.[3] Due to their excellent staining properties, cyanine dyes have been utilized in applications such as flow cytometry,[4] DNA fragment sizing,[5] as reporter groups in hybridization probes,[6–8] single DNA molecule fluorescence microscopy,[9] gel staining,[2] and in real time PCR.[10–12]

Recently, we have developed a series of unsymmetrical cyanine dyes that bind in the minor groove of DNA instead of by intercalation (Fig. 1).[13,14] These dyes have the advantage of large fluorescence quantum yields and absorption at long wavelengths, typical of intercalating cyanine dyes. In addition, their binding in the minor groove is not expected to significantly lengthen the DNA helix, similarly to classical groove-binding DNA stains such as Hoechst 33258 (Hoechst)[15,16] and DAPI.[1,17] Indeed, electrophoresis unwinding assays show that these cyanines do not unwind natural DNA.[14] In agreement with other minor groove binders they have a preference for AT sequences. Whereas Hoechst and DAPI absorb in the near-UV region,[17,18] where background from scattering can be a problem, the absorbance maxima of the groove-bound cyanine dyes are between 465 and 515 nm. A potential disadvantage of the intercalating cyanine dyes is that the insertion of the dye molecules between the base pairs leads to an extension of the DNA helix.[19–21] This might be undesirable in for example studies of hydrodynamic and flexibility properties of DNA in solution, where the polymer length is the crucial variable.[22] Moreover, it has been reported that the minor groove binder DAPI only exhibits a small effect on the DNA conformations

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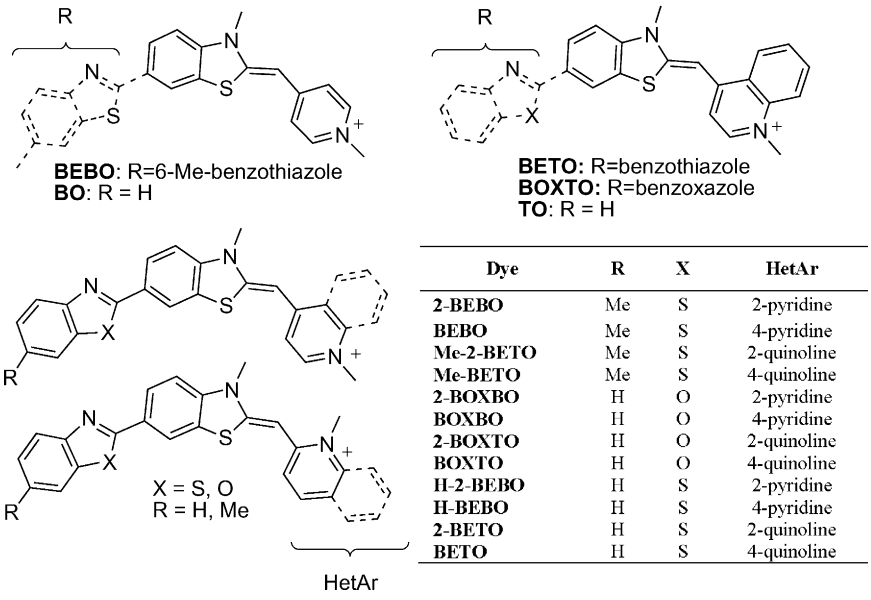


Dye	R	X	HetAr
2-BEBO	Me	S	2-pyridine
BEBO	Me	S	4-pyridine
Me-2-BETO	Me	S	2-quinoline
Me-BETO	Me	S	4-quinoline
2-BOXBO	H	O	2-pyridine
BOXBO	H	O	4-pyridine
2-BOXTO	H	O	2-quinoline
BOXTO	H	O	4-quinoline
H-2-BEBO	H	S	2-pyridine
H-BEBO	H	S	4-pyridine
2-BETO	H	S	2-quinoline
BETO	H	S	4-quinoline

Figure 1. Intercalating (BO and TO), groove binding (BEBO, BETO and BOXTO) and the presently investigated unsymmetrical cyanine dyes (bottom).

but produce less satisfactorily fluorescence images.[20] Whereas many of the intercalating cyanine dyes do not behave very well as reporter dyes in real-time PCR, BEBO has been shown to be a good complement to SYBR Green I as a nonspecific probe in real-time PCR.[10] In addition, minor groove binders are generally more selective toward double than single stranded DNA compared to intercalators.

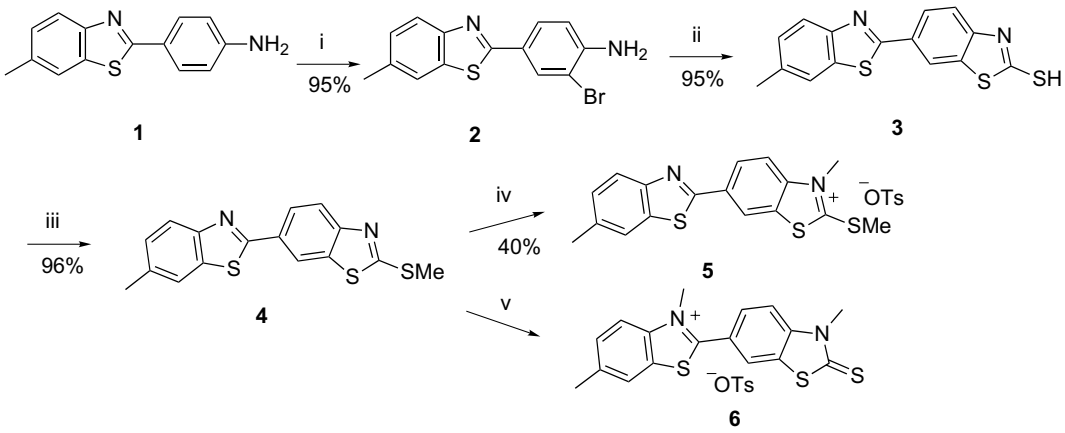
The majority of groove binders interact with DNA by forming hydrogen bonds to the base pairs,[23,24] which greatly stabilize the complex. However, symmetrical cyanine dyes absent of hydrogen bond donors have been found to bind in the minor groove either as monomers[25] or as dimers.[26] Furthermore, Petty and co-workers recently proposed that the unsymmetrical trimethine cyanine dye TO-PRO-3 has a substantial contribution of cooperative minor groove binding to DNA.[27] The dyes prepared by us also lack the capacity of strong hydrogen bonding to poly(dA-dT)$_2$, which only have hydrogen bond acceptors, and studies of these and mentioned cyanine dyes may provide insight to other important parameters for minor groove recognition such as hydrophobicity of the ligands for example.

In BEBO, the structure of the known intercalating dye BO was extended with a 6-methyl-benzothiazole substituent (Fig. 1). The resulting crescent shape of the molecule is similar to that of other minor groove binders such as Hoechst. This structural modification induced a change in binding mode from intercalation toward minor groove binding.[13] The dyes BETO and BOXTO have the same cyanine chromophore as TO but have an extending benzothiazole and benzoxazole group, respectively. BOXTO clearly shows the strongest preference for minor groove binding of the dyes and also the largest quantum yield when bound to DNA (0.52).[14] Both BEBO and BETO show a more heterogeneous binding to mixed sequence DNA than BOXTO, which dominantly binds as monomers in the minor groove. Hence, subtle changes in the structure of the dyes affect the binding to DNA substantially. This motivated us to further investigate how the nature of the different heterocycles in this type of dyes affects their DNA interactions. Herein we present a study of the binding mode to mixed sequence DNA of a number of cyanine dyes, focusing on the effect induced by both changing the heterocycle that is part of the cyanine chromophore as well as the extending heterocyclic moiety (Fig. 1). Three different extending heterocyclic moieties were chosen: benzoxazole, benzothiazole, and 6-methylbenzothiazole. Since BOXTO, having an extending benzoxazole group, exhibits the so far most promising staining properties of these new dyes,[14] further studies of dyes having this extending moiety was motivated. To investigate the significance of the 6-methyl group for the DNA binding mode of the dyes, both benzothiazole and 6-methyl benzothiazole was chosen as extending heterocycles. In addition to the binding studies, a new facile synthetic route to these crescent-shaped cyanine dyes is described.

2. Results and discussion

2.1. Synthesis

The recently reported synthetic strategy toward BEBO and analogous dyes had two problems associated with it.[13] First, a substantial amount of brominated by-product is produced in addition to the desired 2-amino benzothiazole by treatment of the 4-benzothiazolyl aniline **1** with potassium thiocyanate and bromine. Second, the final condensation of 2-imino-3-methyl-6-(6-methyl-benzothiazol-2-yl)-benzothiazoline worked only with 4-methyl pyridinium salts, in modest yields. Here we instead use the classical cyanine dye condensation method, where a thiomethyl group acts as leaving group. The required 2-thiomethyl-benzothiazolium salts



Scheme 1. Reagents and conditions: (i) KBr, $NaBO_3 \cdot 4H_2O$, AcOH, rt, 22 h; (ii) potassium *O*-ethyl dithiocarbonate, DMF, 140 °C, 4 h; (iii) K_2CO_3, iodomethane, DMF, rt, 2 h; (iv) MeOTs (3 equiv), 100 °C, 16 h; (v) MeOTs (large excess), 120 °C, 16 h.

were obtained by a cyclization reaction of the previously undesired 4-aryl-2-bromo-anilines using potassium *O*-ethyl dithiocarbonate yielding 2-thio-benzothiazoles,[28] and subsequent dimethylation.

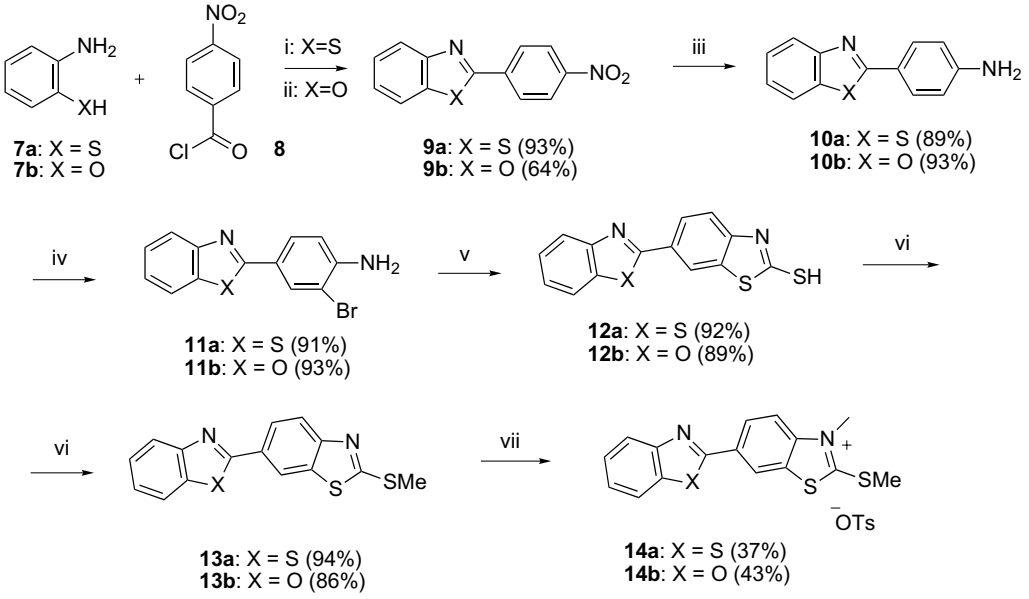
The commercially available aniline **1** was brominated in 95% yield using a recently reported mild oxidative bromination procedure of anilines, where sodium perborate is used as oxidant and potassium bromide as the bromine source (Scheme 1).[29] The thiol **3** was produced in 95% yield by cyclization of the aniline **2** using potassium *O*-ethyl dithiocarbonate in DMF.[28] Treatment of the thiol with methyl iodide in DMF under basic conditions gave the 2-thiomethyl-benzothiazole **4** in 96%.[30] Employing standard procedure, **4** was heated together with excess methyl tosylate at 120 °C to give a monovalent tosylate salt, which, although the NMR spectra (not shown) were consistent with the desired structure **5**, proved to be unreactive toward quaternary salts using the cyanine dye condensation protocol. Thioalkyl groups in the 2-position of benzothiazoles have previously been reported to be quite labile and susceptible to thione formation,[31] and presumably had the thione **6** been formed using the relatively harsh conditions. However, milder conditions (3 equiv neat methyl tosylate at 100 °C) allowed **5** to be isolated in 40% yield.

Several methods yielding 2-aryl-benzothiazoles have been described in the literature. These include condensation of an *ortho*-amino thiophenol with an aromatic aldehyde,[32,33] carboxylic acid,[34] acid chloride,[35] or nitrile.[36] Some of these methods are quite harsh, for example, the condensation with carboxylic acids in polyphosphoric acid at high temperatures.[34,37] There are however milder methods to produce benzothiazoles such as Jacobson oxidative cyclization of thiobenzanilides using alkaline potassium ferricyanide.[38] In a reasonably mild methodology reported by Brembilla et al., *ortho*-amino thiophenol is condensed with various aromatic acid chlorides in *N*-methyl-pyrrolidone (NMP), which acts as a scavenger for the HCl produced in the reaction.[35] Employing this procedure the benzothiazole **9a** was prepared in 93% yield by the condensation of *ortho*-amino thiophenol and 4-nitro-benzoyl chloride **8** (Scheme 2).

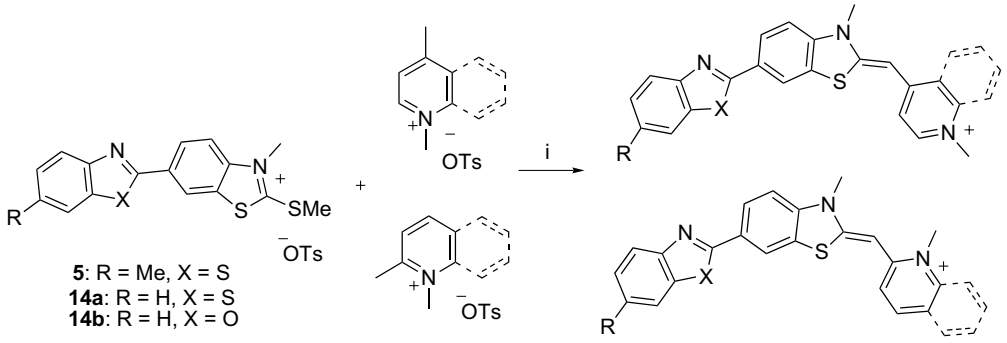
2-Substituted benzoxazoles are usually synthesized by either the condensation of carboxylic acids with 2-aminophenols catalyzed by strong acid[34,37] or by the oxidative cyclization of phenolic Schiff bases using various oxidants.[39–44] The former method requires activation of carboxylic acids under strongly acidic conditions at high temperature. The latter method using DDQ as oxidant was attempted here,[44] but in our hands the condensation of 2-aminophenol and 4-nitro-benzoyl chloride in NMP to produce the benzoxazole **9b** was more convenient. This reaction required longer reaction time than the similar benzothiazole condensation and gave the diacylated (2-hydroxyphenyl)-(4-nitrophenyl)-amide as a by-product. However, the amide was easily removed by trituration with methanol, affording the benzoxazole in 65% yield.

Both nitro compounds **9a**,**b** were reduced using stannous chloride in refluxing ethanol furnishing the anilines **10a** and **10b** in 89% and 93% yield, respectively.[38] The monobrominated anilines **11a**,**b** were attained in over 90% yield using potassium bromide and sodium perborate in acetic acid.[29] Cyclization of the bromo-anilines **11a**,**b** and subsequent dimethylation using the same conditions as in the synthesis of **5** afforded the quaternized salts **14a** and **14b** in a total yield from the starting anilines **7a** and **7b** of 24% and 18%, respectively (for individual yields of the reactions, see Scheme 2).

The quaternary salts **5**, **14a** and **14b** were condensed with quinolinium salts and pyridinium salts with the methyl group in *ortho* or *para* position, thus producing 12 cyanine dyes in total (Scheme 3). The reactions proceeded smoothly in dichloromethane at room temperature using triethyl amine as base.[6] The yields and names of the dyes are summarized in Table 1. In most cases, the *ortho*-methyl salts were more reactive in the condensations than the corresponding salts with *para*-substituted methyl groups. Presumably this is due to the stronger acidity of the methyl groups in 2-position providing a



Scheme 2. Reagents and conditions: (i) NMP, 100 °C, 1 h; (ii) NMP, 100 °C, 17 h; (iii) $SnCl_2{\cdot}2H_2O$, EtOH, 12 h, reflux; (iv) KBr, $NaBO_3{\cdot}4H_2O$, AcOH, rt, 22 h; (v) potassium *O*-ethyl dithiocarbonate, DMF, 140 °C, 4 h; (vi) K_2CO_3, iodomethane, DMF, rt, 2 h; (vii) MeOTs (3 equiv), 100 °C, 16 h.



Scheme 3. Reagents and conditions: (i) NEt_3, CH_2Cl_2, rt, 20 h.

higher concentration of reactive methylene anions in the reaction mixture.

2.2. DNA binding studies

2.2.1. Flow linear dichroism. Linear dichroism (LD) is the difference in absorption of polarized light perpendicular and parallel to an orientation axis. By applying a flow gradient, DNA of sufficient length can become oriented yielding a macromolecular orientation axis. Subsequently, small molecules that bind to DNA in a specific way will also be oriented and their electronic transitions will generate LD signals according to their interaction with DNA.[45] Since the electronic transitions of the DNA bases are perpendicular to the DNA helix axis they will give rise to a negative LD. Thus, intercalating ligands will also have a negative LD whereas minor groove binding compounds have a positive signal. The orientation of the DNA in these studies was achieved using a Couette cell with outer rotating cylinder. 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 Eq. 1. Minor groove binders such as DAPI[46] and Hoechst[47] binds with an angle of around 45° compared to the DNA axis while intercalators binds with an angle close to 90°.[48]

$$LD^r = S \cdot O = \frac{3}{2} \cdot S \cdot (3\cos^2\alpha - 1) \qquad (1)$$

Flow-LD spectra were measured for the dyes in presence of calf thymus DNA (ctDNA) at a mixing ratio, dye:-bases, of 0.025. To illustrate differences in binding mode induced by the extending heterocycle, spectra of dyes having the same cyanine chromophore are jointly presented in the figures. The names of the dyes reflect their basic structure and in order to clarify the following discussions, an explanation of the names of the dyes is motivated. Dyes having a benzoxazole or benzothiazole

Table 1. Yields and extinction coefficients of the dyes synthesized

Dye	R	X	HetAr	Yield (%)	ε^a (M^{-1} cm^{-1})
2-BEBO	Me	S	2-Pyridine	43	33,500
BEBO	Me	S	4-Pyridine	46	32,500
Me-2-BETO	Me	S	2-Quinoline	68	34,000
Me-BETO	Me	S	4-Quinoline	61	41,500
2-BOXBO	H	O	2-Pyridine	81	41,000
BOXBO	H	O	4-Pyridine	40	36,500
2-BOXTO	H	O	2-Quinoline	79	36,000
BOXTO	H	O	4-Quinoline	53	38,000
H-2-BEBO	H	S	2-Pyridine	79	37,000
H-BEBO	H	S	4-Pyridine	47	32,500
2-BETO	H	S	2-Quinoline	89	37,500
BETO	H	S	4-Quinoline	79	38,000

[a] Extinction coefficient of free dyes in 5 mM sodium phosphate buffer (pH 7.0).

group have the prefix BOX or BE, respectively.[†] The suffix of the dyes is dependent on the type of cyanine chromophore and subsequently the parent intercalating dye (TO or BO). Thus, dyes with a pyridinium heterocycle have the suffix –BO and those with a quinolinium group –TO. Finally, dyes having the quaternized nitrogen in the 2-position (2-pyridinium or 2-quinolinium) have the number 2 indicated in the name (see Table 1). To further clarify the presentation of the results, dyes with the same cyanine chromophore will be referred to as 2-pyridinium, 4-pyridinium, 2-quinolinium and 4-quinolinium dyes.

2.2.1.1. 4-Pyridinium dyes. In Figure 2a normalized LD and absorption spectra of dyes with the 4-pyridinium heterocycle (BEBO, H-BEBO, and BOXBO) are shown.[‡] We have previously showed that BEBO have a mixed binding to ctDNA but a significant contribution of minor groove binding.[13] However, the dyes H-BEBO and BOXBO have a more negative LD signal indicating a higher contribution of intercalation. The LD^r spectrum (data not shown) of BOXBO even suggests that intercalation is the major binding mode. It seems that for these dyes the methyl substituent on the benzothiazole moiety in BEBO is important affording a higher degree of minor groove binding.

2.2.1.2. 2-Pyridinium dyes. Figure 2b shows the spectra of DNA complexes of dyes having a 2-pyridinium heterocycle (2-BEBO, H-2-BEBO, and 2-BOXBO). The benzothiazole dyes have a positive LD of a similar shape as that of their isotropic absorption spectra, indicating groove interactions. The LD^r is fairly constant throughout the long wavelength transition for both dyes and, using Eq. 1, the average binding angle is calculated to be 49°. At the absorption band at around 370 nm there are also substantial LD signals. These give rise to a higher LD^r and a corresponding angle of 47°. Interestingly, in this case the methyl group on 2-BEBO does not impose any significant differences in binding compared to that of H-2-BEBO. 2-BOXBO has a very low LD indicating a heterogeneous binding with both intercalation and minor groove binding affording a low LD. However, it could also be due to a substantial amount of randomly oriented dye. However, this is contradicted by results from CD, absorption, and fluorescence measurements.

2.2.1.3. 4-Quinolinium dyes. In contrast to that of BOXBO having a relatively large negative signal, BOXTO has a clear positive LD providing a strong indication of minor groove binding (Fig. 2c). We have studied BOXTO extensively and found it to have the highest preference for monomeric minor groove binding of all unsymmetrical cyanine dyes studied so far.[14] The fact that BOXBO is only one fused benzene ring smaller than BOXTO nicely illustrate the large effect relatively small variations in structure have upon the DNA recognition of these dyes. The other dyes with a 4-quinolinium moiety, BETO and Me-BETO, also have some interesting differences in their LD spectra. BETO has been shown to bind by a combination of monomeric groove binding and as a dimer or a higher oligomer with random orientation. Thus, the second peak in the isotropic absorption spectrum, not present in the LD spectrum, is due to these aggregated nonordered dye molecules. The methyl-substituted dye Me-BETO on the other hand has only a weak LD signal. Symmetrical cyanine dyes, studied by Armitage and co-workers, have been shown to dimerize in the minor groove forming helical aggregates on the DNA template.[26] The degree of dimerization was proposed to be dependent on the hydrophobicity of the dye.[49] Given the higher hydrophobicity of Me-BETO compared to BETO and the insignificant LD signal, randomly ordered dimers might be the dominant interaction of Me-BETO. However, it cannot be ruled out that these dimers are bound in one of the grooves with an angle close to 54°, which would not generate a LD signal either (Eq. 1). Usually, minor groove binding ligands bind with an angle close to 45° though.

2.2.1.4. 2-Quinolinium dyes. In Figure 2d the LD and absorption spectra of the dyes with a 2-quinolinium moiety are shown (Me-2-BETO, 2-BETO, 2-BOXTO). All of these dyes have a strong positive LD with a similar shape to their absorption spectra, indicating minor groove binding. From the LD^r values, the average binding angle at the absorption maxima for the two peaks were calculated using Eq. 1 to be between 43° and 46°. This is similar to the angle for known minor groove binders, for example, DAPI.[46] The peak at shorter wavelength might at the first look be attributed to a band arising from dimers or aggregates, as in the 4-quinolinium case, but is here more probable due to a vibronic progression, as observed for other monomethine cyanine dyes such as YO.[3] The latter explanation

[†] Some of the dyes also have a Me- or H-prefix in order to distinguish them from the already published dyes BEBO, BETO and BOXTO.

[‡] Throughout the entire paper, spectra corresponding to dyes with the benzoxazole, benzothiazole, and 6-methyl-benzothiazole as the extending heterocycle appears in red, blue, and black, respectively.

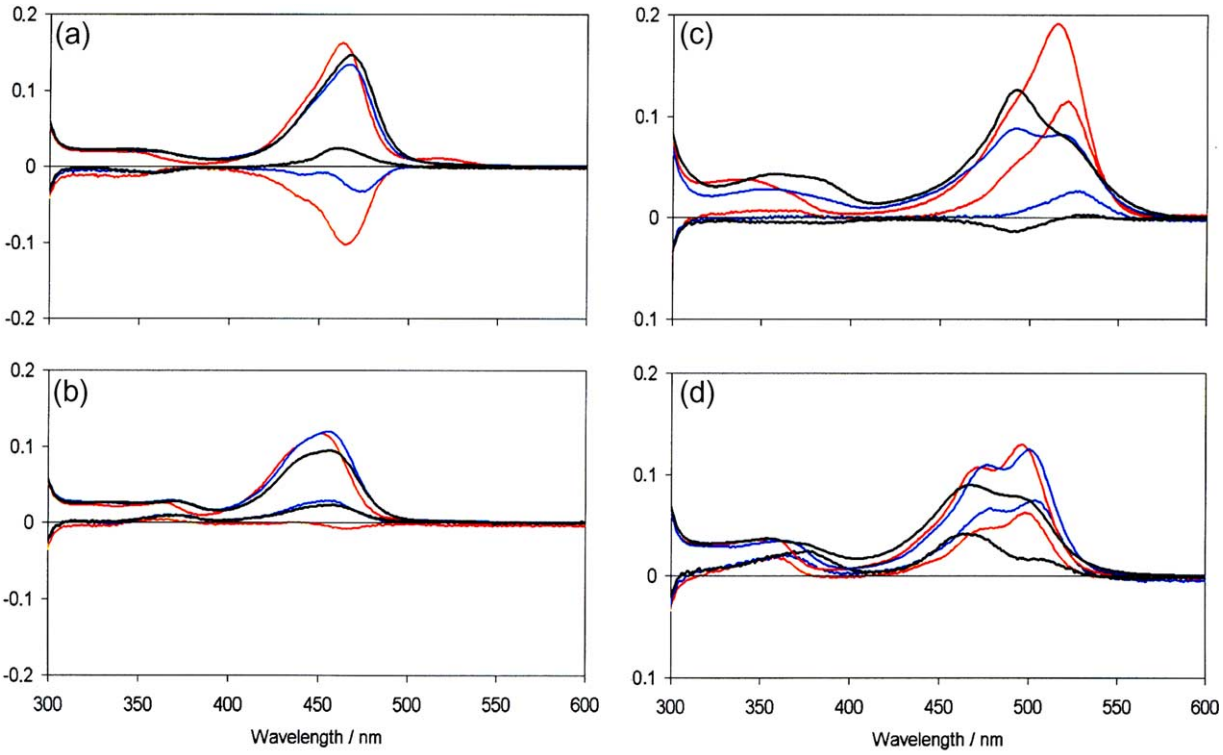


Figure 2. Normalized flow LD and absorption spectra of the cyanine dyes in presence of ctDNA at a mixing ratio, dye:bases, of 1:40, normalized at the DNA base transition. (a) 4-Pyridinium dyes: BEBO (black), H-BEBO (blue), and BOXBO (red); (b) 2-pyridinium dyes: 2-BEBO (black), H-2-BEBO (blue), and 2-BOXBO (red); (c) 4-quinolinium dyes:Me-BETO (black), BETO (blue), and BOXTO (red); (d) 2-quinolinium dyes: Me-2-BETO (black), 2-BETO (blue), and 2-BOXTO (red). The absorption spectra are in all cases the above spectra of the same color. [dye] = 10 μM in all spectra.

is supported by the similar LD and absorption profiles for the benzoxazole and unsubstituted benzothiazole compared to the 6-methyl derivative. For this compound, the most hydrophobic dye Me-2-BETO, the peak at 465 nm is more pronounced than for the other dyes, indicating a higher abundance of dye–dye interactions. Interestingly, the LD^r value for the first band is lower than for the second indicating the presence of intercalating or nonordered monomer dye molecules.

2.2.2. 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 minor groove binders.[50,51] In addition, nonrigid 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.

2.2.2.1. 4-Pyridinium dyes. CD spectra were measured on the same samples as those in the LD measurements (mixing ratio 0.025). In Figure 3a the CD spectra of the dyes with a 4-pyridinium moiety are shown (BEBO, H-BEBO, BOXBO). In agreement with LD results obtained, BEBO has the strongest ICD indicating a higher abundance of minor groove binding. BOXBO, which has the most negative LD of all the dyes studied in this series, also have a relatively low CD signal further supporting that the dominant interaction is by intercalation.

2.2.2.2. 2-Pyridinium dyes. Figure 3b shows the CD spectra of DNA complexes of dyes having a 2-pyridinium heterocycle (2-BEBO, H-2-BEBO, and 2-BOXBO). As in the LD measurements, the CD spectra of the two dyes with a benzothiazole moiety are similar with a relatively large ICD. Although 2-BOXBO only had a very weak LD signal, the ICD indicates that there is a relatively high abundance of groove bound dye molecules. In comparison to the 4-pyridinium dyes, the ICD at the absorption band at around 370 nm is much larger for these dyes. The reason for this is not clear, although it might be due to that the extending heterocycle is rotated slightly out of co-planarity by the groove interactions, affording a chiral conformation of the bis-benzazole moiety. However, it could be noted that also rigid heterocycles can obtain very strong ICD when bound in the minor groove.[52] Interestingly, the large ICD at 370 nm of the 2-pyridinium dyes is comparable to that at 350 nm of the bis-benzimidazole dye Hoechst,[53] indicating that the bis-benzazole and bis-benzimidazole chromophores may be bound in a similar way.

2.2.2.3. 4-Quinolinium dyes. In Figure 3c the CD spectra of the 4-quinolinium dyes in presence of ctDNA

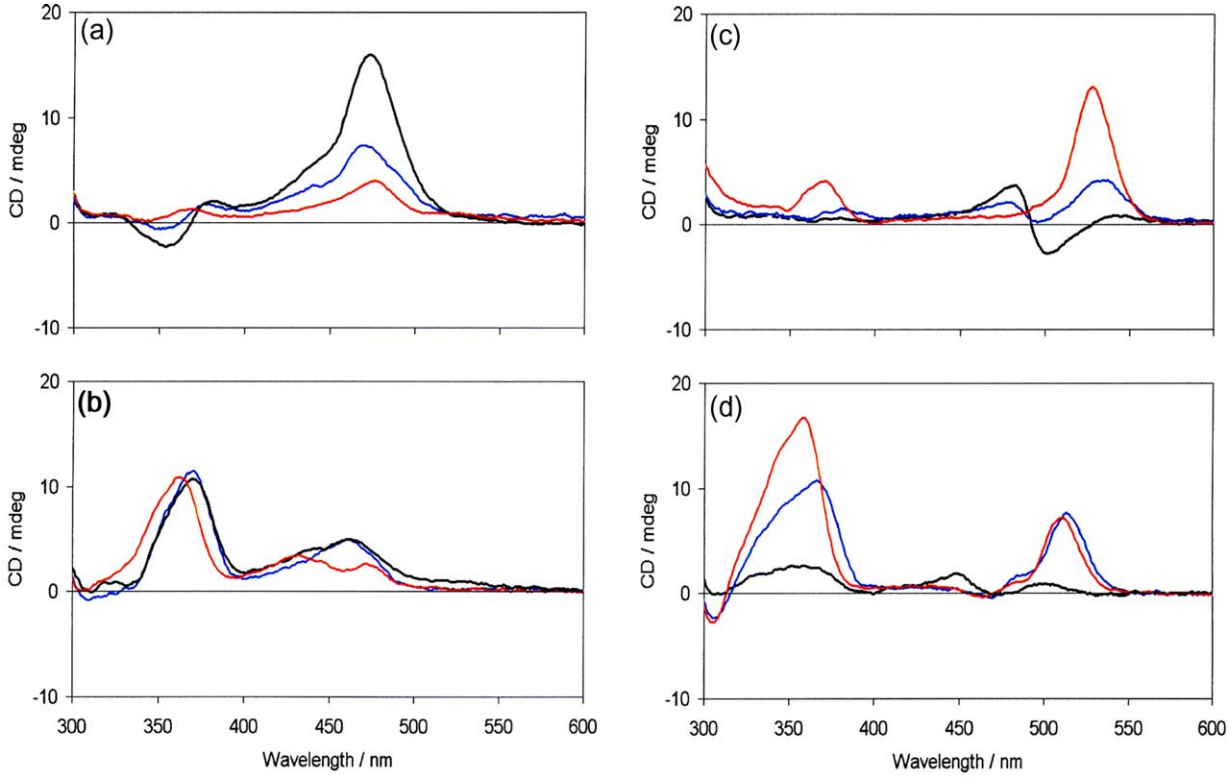


Figure 3. CD spectra of the cyanine dyes in presence of ctDNA at a mixing ratio, dye:bases, of 1:40, normalized at the DNA base transition. (a) 4-Pyridinium dyes: BEBO (black), H-BEBO (blue), and BOXBO (red); (b) 2-pyridinium dyes: 2-BEBO (black), H-2-BEBO (blue), and 2-BOXBO (red); (c) 4-quinolinium dyes: Me-BETO (black), BETO (blue), and BOXTO (red); (d) 2-quinolinium dyes: Me-2-BETO (black), 2-BETO (blue), and 2-BOXTO (red), [dye] = 10 μM in all spectra.

are shown. BOXTO has an ICD of large amplitude further supporting groove binding. The stronger preference for monomeric groove binding by BOXTO is illustrated by roughly a twice as large ICD of BOXTO than to 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. The higher hydrophobicity of Me-BETO relative to BETO might imply that the driving force for dimerization of Me-BETO upon interaction with DNA is larger. Indeed, the CD spectrum shows an exciton-coupled signal around 490 nm as the major signal, indicating randomly bound dimers as the dominant interaction.

2.2.2.4. 2-Quinolinium dyes. In Figure 3d the CD spectra of the dyes with a 2-quinolinium moiety are shown. For both 2-BOXTO and 2-BETO the ICD at the long wavelength transition is relatively large supporting minor groove binding. In contrast, the ICD of Me-2-BETO is very low at this wavelength. This is in agreement with the LD results (Fig. 2d), which shows that the contribution of groove-bound monomers of Me-2-BETO is smaller than that of the other dyes with the same cyanine chromophore. According to the absorption and LD measurements Me-2-BETO has a high abundance of dimer interaction with ctDNA. The dimer absorption peak of Me-2-BETO has its maximum at around 465 nm, where the cross-over of the exciton couplet should be found. However, the red-shifted band of the CD couplet is possibly cancelled out due to the positive sign of the ICD from bound monomer dye molecules at the same wavelength.

Similarly to the dyes with the 2-pyridinium moiety, the 2-quinolinium series of dyes have a large ICD at the short wavelength transition band around 370 nm (see above).

2.2.3. Absorption measurements. A common feature of cyanine dyes is their tendency to aggregate in water solutions.[54] Dimerization and further aggregation of cyanine dyes upon binding to DNA has also been observed in a number of studies.[26,49,55,56] In Figures 5–8 are shown comparisons between the dyes absorption spectra in presence of ctDNA at a mixing ratio 1:40 (a) and in methanol (b), where the dye molecules are presumably present as monomers.[13,54,57]

2.2.3.1. 2- and 4-Pyridinium dyes. In Figure 4a, b spectra of dyes having a 4-pyridinium moiety are shown. The shape of the spectra in methanol and in presence of DNA is quite similar indicating that most of the dyes are bound as monomers. In the absorption spectra of the dyes with a 2-pyridinium moiety in presence of ctDNA, there is a small shoulder at shorter wavelengths, which

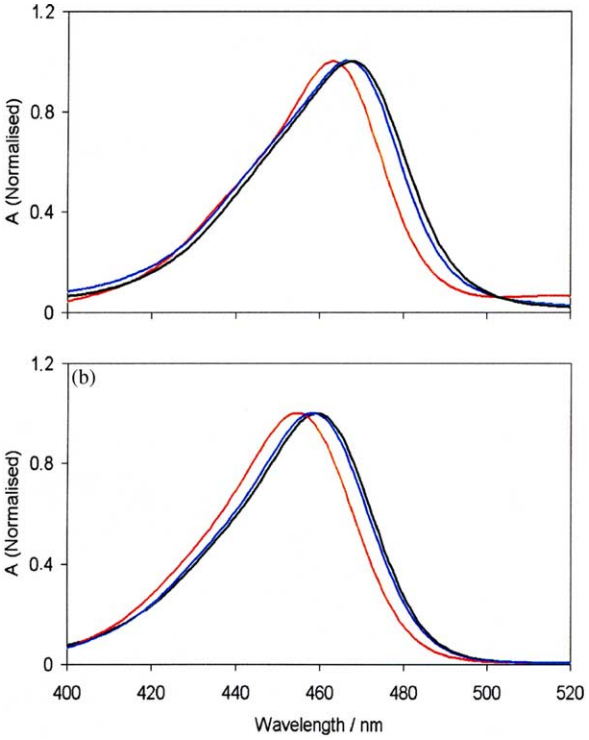


Figure 4. Normalized absorption spectra of BEBO (black), H-BEBO (blue), and BOXBO (red) in presence of ctDNA at a mixing ratio, dye:bases, of 1:40 (a) and free in methanol solution (b).

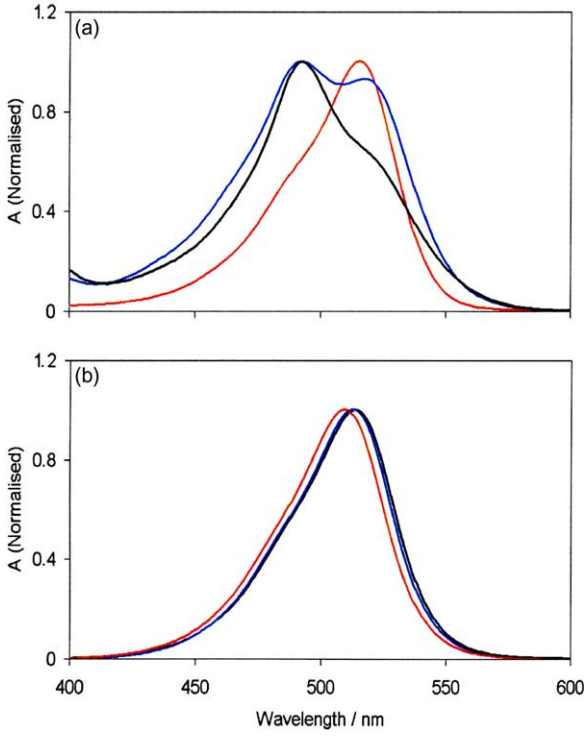


Figure 6. Normalized absorption spectra of Me-BETO (black), BETO (blue), and BOXTO (red) in presence of ctDNA at a mixing ratio, dye:bases, of 1:40 (a) and free in methanol solution (b).

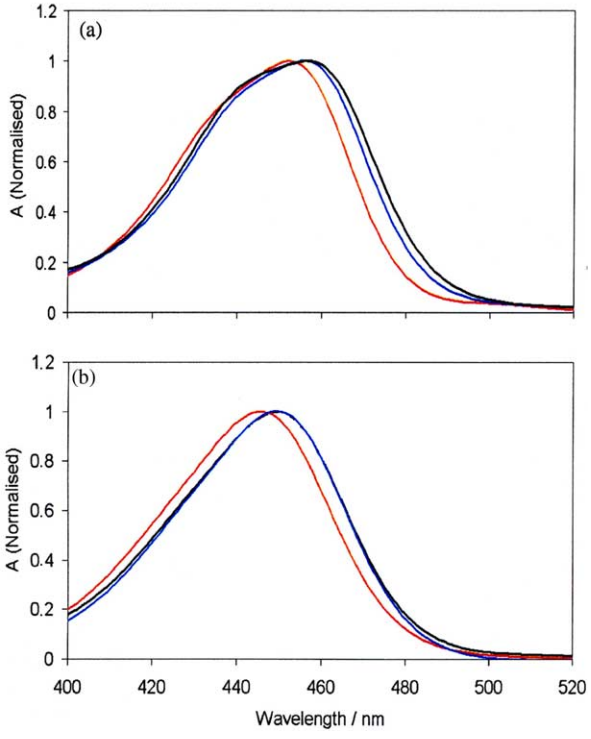


Figure 5. Normalized absorption spectra of 2-BEBO (black), H-2-BEBO (blue), and 2-BOXBO (red) in presence of ctDNA at a mixing ratio, dye:bases, of 1:40 (a) and free in methanol solution (b).

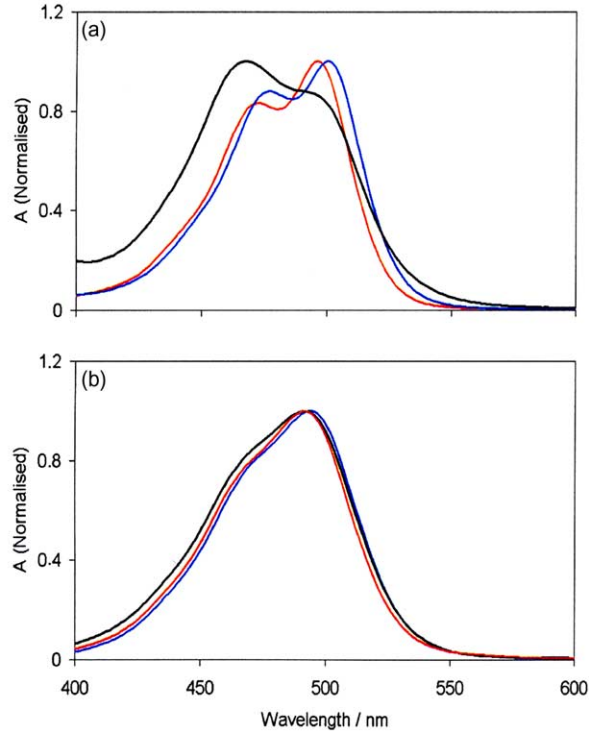


Figure 7. Normalized absorption spectra of Me-2-BETO (black), 2-BETO (blue), and 2-BOXTO (red) in presence of ctDNA at a mixing ratio, dye:bases, of 1:40 (a) and free in methanol solution (b).

might be due to the presence of dimers (Fig. 5). However, the same shoulder is also observed in the LD spectra and since the LD^r (data not shown) is reasonably constant throughout this transition it is likely that

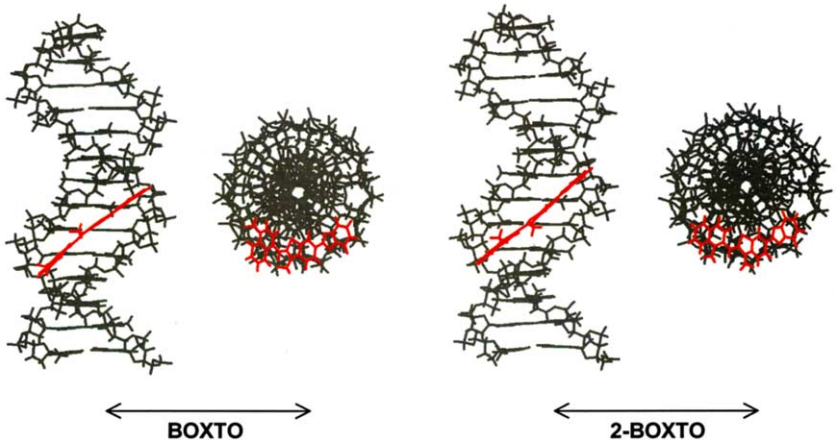


Figure 8. Energy minimized structures of BOXTO (left) and 2-BOXTO (right) docked into poly(dA-dT)$_2$.

it originates from the same electronic transition and that the shoulder correspond to different vibronic transitions to the same electronic excited state.[3]

2.2.3.2. 4-Quinolinium dyes. Figure 6 shows the absorption spectra of the 4-quinolinium containing dyes. Interestingly, the most hydrophobic dye, Me-BETO, shows the highest degree of dimer interaction with DNA, illustrated by the dominant dimer peak at 490 nm. The dimeric interaction is also supported by CD results, showing an exciton-coupled signal at 490 nm (Fig. 3c). BETO clearly exhibits a substantial dimeric binding, whilst the least hydrophobic dye, BOXTO, binds predominantly as a monomer.

2.2.3.3. 2-Quinolinium dyes. In Figure 7 the similar absorption spectra as above are shown for dyes with a 2-quinolinium heterocycle. Analogous to that of the 4-quinolinium series, the most hydrophobic dye with a 6-methyl-benzothiazole moiety (Me-2-BETO) has a dominant dimer peak at around 465 nm. In case of 2-BETO and 2-BOXTO, there is also another peak at shorter wavelengths, which is similar to that of the 2-pyridinium dyes are also present in the LD spectra. Since there is a shoulder observed in the methanol spectra at similar wavelengths, which again probably originate from other vibronic transitions within the same electronic excited state,[3] this suggests that the short wavelength peak in the presence of DNA more strongly correspond to different vibronic transitions rather than bound dimers. In addition, these peaks are slightly red-shifted in comparison with the presumed dimer peak of Me-2-BETO. Upon binding to DNA the dyes will become more rigid, which might explain the more distinct vibronic peaks in the DNA spectra, compared to the spectra of free dye in methanol.

Table 2. Absorption, fluorescence, and binding properties of the investigated dyes bound to ctDNA[a]

Dye	Ext. HetAr[b]	$\lambda_{abs.\ max.}$ (nm)	$\lambda_{ems.\ max.}$ (nm)	ϕ_F[c]	ϕ_{bound}/ϕ_{free}	F_{bound}/F_{free}[d]	LD^r_{dye}/LD^r_{DNA}[e]	Main binding mode[f]/main bound form[g]
BEBO	Me-Bt.	467	492	0.18	16	300	−0.15	Intermediate/monomer
2-BEBO	Me-Bt.	455	502	0.062	7	60	−0.29	(Minor groove)[h]/monomer
Me-BETO	Me-Bt.	493	570	0.086	6	40	+0.002	Intermediate/dimer
Me-2-BETO	Me-Bt.	465	550	0.079	9	100	−0.48	Minor groove/dimer
H-BEBO	Bt.	467	491	0.19	6	300	+0.20	Intermediate/monomer
H-2-BEBO	Bt.	454	495	0.081	15	200	−0.24	(Minor groove)[h]/monomer
BETO	Bt.	516	561	0.21	14	130	−0.24	(Minor groove)[h]/(dimer) [h,i]
2-BETO	Bt.	500	542	0.22	24	180	−0.59	Minor groove/monomer
BOXBO	Box.	463	487	0.41	32	340	+0.61	Intercalation/monomer
2-BOXBO	Box.	452	488	0.082	30	100	+0.017	Intermediate/monomer
BOXTO	Box.	515	552	0.52	50	260	−0.60	Minor groove/monomer
2-BOXTO	Box.	495	535	0.32	72	370	−0.58	Minor groove/monomer

[a] Measured at 25 °C in 5 mM sodium phosphate buffer (pH 7.0) at a mixing ratio, dye:bases, of 1:100.
[b] Extending heterocycle on the cyanine chromophore. Me-Bt., Bt., and Box. correspond to methyl-benzothiazole, benzothiazole, and benzoxazole, respectively.
[c] Fluorescence quantum yields, ϕ_F were determined relative to fluorescein in 0.1 M NaOH, assuming a ϕ_F of 0.93.
[d] F_{bound}/F_{free} is the largest increase in fluorescence intensity (dependent on wavelength) upon binding to DNA.
[e] The ratio between the reduced LD at $\lambda_{abs.\ max.}$ and at the DNA transition (260 nm). A ratio of −0.50 corresponds to an average binding angle of 45° (minor groove binding) and a ratio of +1.0 an angle of 90° (intercalation) (from Eq. 1). Dye:bases ratio 1:40.
[f] The main binding mode as deduced from LD and CD results. Dye:bases ratio 1:40.
[g] The main bound form of the dyes to ctDNA, roughly estimated from absorption and CD results.
[h] The brackets note a slight majority of the designated binding interaction.
[i] The minor groove interaction of BETO is dominated by monomers.[14]

2.2.4. Fluorescence measurements. The fluorescence properties of the dyes in presence of ctDNA at a mixing ratio, dye:bases, of 1:100 are shown in Table 2. The relatively high quantum yield of many of these cyanines bound to DNA combined with their absorption at long wavelengths may render them useful in biological applications. Although some of these dyes bind by a combination of groove binding and intercalation they may still be useful as nonselective stains for DNA due to their large quantum yield when bound. For example, BOXBO has a quantum yield of 0.41, which can be compared to 0.083 for the parent intercalating dye BO.[58] As a further comparison, the monovalent intercalating cyanine dye TO has a quantum yield of 0.11,[55] and the tetravalent cyanine dyes YOYO and TOTO of around 0.4 when bound to DNA.[59] Both the 2-pyridinium and the 2-quinolinium dyes have a slightly lower absorption and emission maximum than the corresponding *para*-substituted cyanines, affording a possibly useful extension of the spectral characteristics of this type of cyanine dyes.

For all new dyes, a blue-shift in emission maxima upon binding to DNA of between 30 and 70 nm is observed (data not shown). This blue-shift can be of advantage since the fluorescence intensity for the free dye at wavelengths near the emission maxima for the bound dye is low, resulting in a larger enhancement in fluorescence intensity when measured at shorter wavelengths compared to the total increase in quantum yield. The increase in quantum yield of the dyes upon binding to DNA varies between 6- and 72-fold, whereas the maximum increase in fluorescence intensity is between 40 and 370. The most prominent monomeric minor groove binder of the presently investigated dyes, BOXTO, also has the largest quantum yield when bound to ctDNA (0.52). The benzoxazole containing dye 2-BOXTO also has a high quantum yield, 0.32, and the largest increase in quantum yield upon binding.

2.3. Implications of structural influence on binding mode

In order to facilitate discussions of the relative degree of groove binding of these dyes, the ratios between the LD^r value of the cyanine transitions and that of the DNA base transitions are shown in Table 2 (also known as the dichroism ratio parameter).[47] A value of −0.5 correspond to an angle of 45° relative to the DNA helix axis (Eq. 1), which is characteristic of minor groove binding ligands. Purely intercalating compounds would have a ratio close to +1.0, which corresponds to the transition moment of the ligand being oriented completely parallel to the DNA bases. In the following discussion it is assumed that lower LD signals, affording a less negative or a positive ratio, for some of the dyes are due to a contribution of either intercalated or nonoriented externally bound dye. Alternatively, oriented dye binding in the minor groove with an angle of 54° would have no LD and thus also lead to a decrease in the total LD signal. This is less likely however, since BEBO, BOXTO, and BETO bind in the minor groove of poly(dA-dT)$_2$ with an angle of 45°.[13,14] In addition, many of these dyes bind with an angle close to 45° to mixed sequence ctDNA.

The 2-pyridinium dyes have a higher proportion of groove binding than the dyes with the 4-pyridinium dyes. Similarly, the 2-quinolinium dyes are principally bound in the groove and have a larger preference for minor groove binding than the 4-quinolinium dyes, with the exception of BOXTO. The thermodynamically most stable conformations of these dyes would have the methylated nitrogen directed out from the groove (Figs. 1 and 8). This might impose less steric hindrance and stronger van der Waals interactions with the walls of the groove. In addition, the 2-quinolinium dyes will have the fused benzene ring in a different direction compared to the 4-quinolinium dyes, affording a longer crescent-shaped dye with a slight difference in overall curvature. The requirement of isohelicity of minor groove binding ligands have been highlighted in several studies[60–62] and the 2-quinolinium dyes may be more isohelical with the minor groove than the 4-quinolinium dyes. Indeed, energy minimized structures of BOXTO and 2-BOXTO docked into an alternating AT oligomer indicate that the fused benzene ring of 2-BOXTO follows the groove, whereas in BOXTO this ring is pointing outwards (Fig. 8). However, both dyes fit very well in the groove, covering roughly 4 AT base pairs. The delocalization of charge will also vary between the different types of cyanine chromophores. One could only speculate to what extent each of these parameters will contribute to the affinity for the minor groove and ultimately crystal or NMR structures of the dye–DNA complexes is warranted.

The variation of the extending benzazole heterocycle also provides some curious differences in binding mode. The significance of the methyl group on the benzothiazole ring is more apparent in the case of the 4-pyridinium than the 2-pyridinium dyes, illustrated by the higher degree of groove binding of BEBO than H-BEBO lacking the methyl group and the negligible difference between 2-BEBO and H-2-BEBO. Possibly, steric hindrance toward the minor groove induced by the methyl group in BEBO forces the nitrogen atom on the benzothiazole moiety to be directed into the groove. This might stabilize the complex to a higher degree than when free rotation of the benzothiazole group allows the sulphur to be directed inwards. Alternatively, it might be a hydrophobic effect. In a study by Mikheikin et al., trimethine symmetrical cyanine dyes were found to bind in the minor groove as monomers.[25] They proposed that the DNA-binding ligands should be sufficiently hydrophobic to provide a high affinity for the minor groove. For the 4-pyridinium dyes this seems to fit reasonably well: the more hydrophobic BEBO has the strongest affinity for the minor groove and BOXBO being the least hydrophobic binds with a high degree of intercalation. However in the case of 2-BEBO and H-2-BEBO, which interact with DNA in a very similar fashion, the methyl group is nonsignificant. In the 4-quinolinium dyes the methyl group induce an increase of the abundance of dimer interaction. This is most evident in the case of Me-BETO, where the dimer interactions clearly

dominate (Table 2). The benzothiazole dye BETO has a faint majority of dimer interactions, whereas BOXTO binds mainly as a monomer. It seems that sufficient hydrophobicity and size is crucial for monomeric recognition of the minor groove of these dyes. However, slight increases in hydrophobicity cause the dyes to bind in a dimeric fashion.

3. Conclusions

A new facile synthetic route to a number of crescent-shaped unsymmetrical dyes has been developed. The yields of the reactions toward the bis-benzazoles **4** and **13a**,**b** are good to excellent. The quaternizations of the benzothiazole nitrogen proceeds in relatively modest yields, whereas the cyanine dyes condensations proceeds in satisfactorily yields. Flow LD and CD studies of the 12 dyes show that the extent of minor groove binding to mixed sequence ctDNA varies between the dyes. The 2-quinolinium dyes (Me-2-BETO, 2-BETO, and 2-BOXTO) bind principally in the minor groove both as monomers and as dimers (Me-2-BETO). In the case of the 4-quinolinium dyes (Me-BETO, BETO, and BOXTO), nonordered dimeric interactions increase with the hydrophobicity of the dyes. BOXTO exhibits the most pronounced monomeric minor groove binding of the dyes studied as yet. The 2-pyridinium dyes (BEBO, H-BEBO, and BOXBO) have a higher contribution of intercalation going from higher to lower hydrophobicity. However, this increase in the degree of intercalation can be a pure structural effect and also be dependent on the size of the dyes. In BEBO the methyl group on the extending benzothiazole moiety might impose a more favorable conformation for minor groove binding. Relative to the 4-pyridinium dyes, the 2-pyridinium dyes (2-BEBO, H-2-BEBO, and 2-BOXBO) have a higher contribution of minor groove binding.

The relatively high fluorescence quantum yield of many of these cyanines bound to DNA combined with their absorption at long wavelengths may render them useful in biological applications. In particular, two of the benzoxazole containing dyes BOXTO and 2-BOXTO show a high degree of minor groove binding and quantum yields of 0.52 and 0.32, respectively, when bound to DNA. Upon binding to ctDNA the dyes exhibit varyingly large enhancements in quantum yield. The lowest increase is observed for Me-BETO and the largest for 2-BOXTO, with a 6-fold and a 72-fold increase, respectively. In most cases, dyes with a larger contribution of dimer interaction have a lower quantum yield when bound to DNA. Due to a blue-shift in emission maxima upon binding to DNA, the increase in fluorescence intensity are larger than the quantum yield enhancements when measured at slightly shorter wavelengths than the emission maxima and varies between 40- and 370-fold. Finally, the introduction of cyanine chromophores having the quinolinium or pyridinium moiety in 2-position provide a possibly useful extension of the spectral characteristics of this type of cyanine dyes.

4. Experimental

4.1. Spectroscopic measurements

UV–vis spectra were measured on a Varian Cary4 spectrophotometer. Fluorescence spectra were recorded using a SPEX fluorolog $\tau 2$ 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 to 3 mM stock solutions in DMSO. The DNA concentration per nucleotide was determined by absorption spectroscopy using extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm. The cyanine dye concentrations were estimated using the extinction coefficients found in Table 1. Calf thymus DNA was purchased from Fluka.

4.2. Mathematical modeling

Structures of 2-BOXTO and BOXTO were optimized with PM3, docked into the minor groove of an alternating AT oligomer in canonical B-DNA conformation, and further energy minimized in the MM+ force field of the HYPERCHEM software package (HyperCube, Inc.).

4.3. Synthesis

Flash and column chromatography was performed using aluminium oxide (activated, neutral, approx. 150 mesh), deactivated by addition of water to Brockman grade III, and silica gel (Merck, grade 60, 70–230 mesh). ^1H (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-d_6 (δ 2.50 for ^1H and δ_C 39.51 for ^{13}C NMR), $CDCl_3$ (δ_{TMS} 0 for ^1H δ_C 77.0 for ^{13}C NMR) and methanol-d_4 (δ 3.31 for ^1H and δ_C 49.0 for ^{13}C NMR). J values are given in Hertz. High resolution mass spectra were performed with a VG 7070E magnetic sector instrument (VG Analytical/Micromass, Manchester, UK). Conditions for FAB (fast atom bomardment): Xe gun at 8 kV, matrix glycerol or 3-nitrobenzylalcohol with PEG600 as mass reference. A signal from a coil in the magnet field was used for mass calibration. Commercial reagents were purchased from Sigma-Aldrich and used without further purification. 2-(4-Aminophenyl)benzothiazole **10a** was prepared from 2-aminothiophenol **7a** in two steps as previously described.[35,38]. 2-(4-Nitrophenyl)benzoxazole **9b** was reduced according to a previously reported procedure to afford 2-(4-aminophenyl)benzoxazole **10b** in 93% yield.[63] Potassium *O*-ethyl dithiocarbonate[64] and 4-nitrobenzoyl chloride[65] were prepared as described previously.

4.3.1. 2-(4-Nitrophenyl)benzoxazole 9b. 4-Nitrobenzoylchloride (2.25 g, 15 mmol) was added in portions to a

solution of 2-aminophenol (1.63 g, 15 mmol) in dry NMP (10 mL) (caution, exothermic reaction). The mixture was stirred at 100 °C for 17 h. After cooling, the mixture was poured into water and the pH was adjusted to 8–9 by the addition of aqueous ammonia. The precipitate formed was collected by filtration, washed with methanol, and air dried to afford **9b** as a white solid (2.31 g, 64%).^{1}H NMR ($CDCl_3$): δ 7.40–7.48 (2H, m, ArH), 7.65 (1H, d, $J = 8$, ArH), 7.84 (1H, d, $J = 8$, ArH) 8.36–8.50 (4H, m, ArH). These data are consistent with those reported for the previously prepared compound.[66]

4.3.2. General procedure for bromination to afford 2 and 11a,b. $NaBO_3{\cdot}4H_2O$ (3.38 g, 22 mmol) was added in one portion to a suspension of the appropriate aniline (20 mmol) and KBr (2.86 g, 24 mmol) in concentrated acetic acid (15 mL). After stirring at rt for 22 h, the reaction was quenched by the addition of ice water. The precipitate formed was collected by filtration, washed with water, and air dried to afford the *ortho*-brominated aniline.

4.3.2.1. 2-(4-Amino-3-bromophenyl)-6-methyl-benzothiazole 2. Obtained from the aniline **1** (4.81 g, 20 mmol) as a white solid (6.07 g, 95%) using the above-described procedure. ^{1}H NMR (DMSO-d_6): δ 2.43 (3H, s, Ar-CH_3), 6.07 (2H, s, NH_2), 6.89 (1H, d, $J = 8$, ArH), 7.30 (1H, d, $J = 8$, ArH), 7.74 (1H, dd, $J_1 = 8$, $J_2 = 2$, ArH), 7.81 (1H, d, $J = 8$, ArH), 7.84 (1H, s, ArH), 8.02 (1H, d, $J = 2$, ArH). These data are consistent with those reported for the previously prepared compound.[38]

4.3.2.2. 2-(4-Amino-3-bromophenyl)benzothiazole 11a. Obtained from the aniline **1** (4.53 g, 20 mmol) as a white solid (5.55 g, 91%) using the above-described procedure. ^{1}H NMR ($CDCl_3$): δ 4.45 (2H, s, NH_2), 6.83 (1H, d, $J = 8$, ArH), 7.35 (1H, d, $J = 8$, ArH), 7.47 (1H, d, $J = 8$, ArH), 7.82 (1H, d, $J = 8$, ArH), 7.87 (1H, d, $J = 8$, ArH), 8.0 (1H, d, $J = 8$, ArH), 8.21 (1H, s, ArH). These data are consistent with those reported for the previously prepared compound.[38]

4.3.2.3. 2-(4-Amino-3-bromophenyl)benzoxazole 11b. Obtained from the aniline **9b** (4.20 g, 20 mmol) as a white powder (5.38 g, 93%) using the above-described procedure. ^{1}H NMR ($CDCl_3$): δ 4.51 (2H, s, NH_2) 6.86 (1H, d, $J = 8$, ArH), 7.33 (2H, m, ArH), 7.54 (1H, d, $J = 8$, ArH), 7.71 (1H, d, $J = 8$, ArH), 8.00 (1H, d, $J = 8$, ArH), 8.35 (1H, s, ArH); ^{13}C NMR (DMSO-d_6): δ 106.7, 110.5, 114.5, 115.0, 119.1, 124.6, 127.8, 127.9, 131.4, 141.8, 149.3, 149.9, 162.0; HR-FAB-MS m/z found 289.001, $C_{13}H_9BrN_2O$ (M+H$^+$): requires M, 289.006.

4.3.3. General procedure for preparation of 2-mercaptobenzothiazoles 3 and 12a,b. To a solution of the appropriate bromoaniline (19 mmol) in anhydrous DMF (30 mL) was added potassium *O*-ethyl dithiocarbonate (6.8 g, 42 mmol) in portions. The mixture was heated to 140 °C for 4 h. After cooling the mixture was diluted with water (30 mL) and acidified by 6 N HCl. The precipitate formed was collected by filtration, washed with water and dried to obtain the thiol as a faint yellow solid.

4.3.3.1. 6-(6-Methyl-benzothiazol-2-yl)-2-mercaptobenzothiazole 3. Obtained from the bromo-aniline **2** (6.05 g, 19 mmol) using the above-described procedure (5.68 g, 95%). ^{1}H NMR (DMSO-d_6): δ 2.45 (3H, s, Ar-CH_3), 7.35 (1H, d, $J = 8$, ArH), 7.41 (1H, d, $J = 8$, ArH), 7.90 (2H, m, ArH), 8.08 (1H, d, $J = 8$, ArH), 8.42 (1H, s, ArH); ^{13}C NMR (DMSO-d_6): δ 21.15, 112.9, 120.6, 121.9, 122.3, 126.4, 128.2, 129.0, 130.6, 134.6, 135.4, 143.3, 151.7, 165.3, 191.0; HR-FAB-MS m/z found: 315.001 $C_{15}H_{11}N_2S_3$ (M+H$^+$): requires M, 315.0084.

4.3.3.2. 6-(Benzothiazol-2-yl)-2-mercaptobenzothiazole 12a. Obtained from the bromo-aniline **11a** (5.80 g, 19 mmol) using the above-described procedure (5.25 g, 92%). ^{1}H NMR (DMSO-d_6): δ 7.46 (2H, m, ArH), 7.55 (1H, d, $J = 8$, ArH) 8.03 (1H, d, $J = 8$, ArH), 8.14 (2H, m, ArH) 8.48 (1H, s, ArH), 14.02 (1H, s, SH); HR-FAB-MS m/z found: 301.422 $C_{14}H_9N_2S_3$ (M+H$^+$): requires M, 301.430.

4.3.3.3. 6-(Benzoxazol-2-yl)-2-mercaptobenzothiazole 12b. Obtained from bromo-aniline **11b** (5.49 g, 19 mmol) using the above-described procedure (4.81 g, 89%). ^{1}H NMR (DMSO-d_6): δ 4.32 (1H, s, SH), 7.43 (2H, m, ArH), 7.48 (1H, d, $J = 8$, ArH) 7.79 (1H, dd, $J_1 = 8$, $J_2 = 2$, ArH), 8.22 (1H, d, $J = 8$, ArH) 8.57 (1H, s, ArH); ^{13}C NMR (DMSO): δ 110.8, 113.0, 119.8, 120.9, 122.2, 124.9, 125.5, 126.6, 130.5, 141.5, 143.8, 150.2, 161.8, 191.2; HR-FAB-MS m/z found: 284.001 (M$^+$): $C_{14}H_8N_2OS_2$ requires M, 284.008.

4.3.4. General procedure for preparation of 2-thiomethyl benzothiazoles 4 and 13a,b. To the appropriate 2-mercapto benzothiazole (16 mmol) and potassium carbonate (3.3 g, 24 mmol) in anhydrous DMF (50 mL) was added iodomethane (2.71 g, 19 mmol). After stirring at rt for 2 h water (50 mL) was added and the precipitate formed was collected by filtration, washed with water, and air dried. The analytical samples were recrystallized from ethyl acetate to give the benzothiazoles as a faint yellow solids.

4.3.4.1. 6-(6-Methyl-benzothiazol-2-yl)-2-thiomethyl-benzothiazole 4. Obtained from the mercaptobenzothiazole **3** (5.0 g, 16 mmol) using the above-described procedure (5.04 g, 96%). %). ^{1}H NMR (DMSO-d_6): δ 2.46 (3H, s, Ar-CH_3), 2.83 (3H, s, S-CH_3), 7.37 (1H, d, $J = 8$, ArH), 7.95 (3H, m, ArH), 8.14 (1H, d, $J = 8$, ArH), 8.79 (1H, s, ArH); HR-FAB-MS m/z found: 329.032 $C_{16}H_{13}N_2S_3$ (M+H$^+$): requires M, 329.0241.

4.3.4.2. 6-(Benzothiazol-2-yl)-2-thiomethyl-benzothiazole 13a. Obtained from the mercaptobenzothiazole **12a** (4.81 g, 16 mmol) using the above-described procedure (4.73 g, 94%). ^{1}H NMR (DMSO-d_6): δ 2.84 (3H, s, S-CH_3), 7.48 (1H, t, $J = 8$, ArH), 7.56 (1H, t, $J = 8$, ArH), 7.99 (1H, d, $J = 8$, ArH), 8.07 (1H, d, $J = 8$, ArH), 8.18 (2H, m, ArH), 8.84 (1H, s, ArH); HR-FAB-MS m/z found: 315.013 $C_{15}H_{11}N_2S_3$ (M+H$^+$): requires M, 315.0084.

4.3.4.3. 6-(Benzoxazol-2-yl)-2-thiomethyl-benzothiazole 13b. Obtained from the mercaptobenzothiazole **12b** (4.55 g, 16 mmol) using the above-described procedure (4.1 g, 86%). ^{1}H NMR ($CDCl_3$): δ 2.85 (3H, s, S-CH_3), 7.38 (1H, d, $J_1 = 8$, $J_2 = 2$, ArH) 7.60 (1H, t, $J = 8$, ArH), 7.78 (1H, t, $J = 8$, ArH), 7.98 (1H, d, $J = 8$, ArH), 8.33 (1H, d, $J = 8$, ArH), 8.69 (1H, s, ArH); ^{13}C NMR ($CDCl_3$): δ 16.22, 110.8, 120.2, 120.7, 121.8, 123.1, 124.9, 125.3, 125.8, 136.0, 142.4, 151.0, 155.5, 162.8, 171.6; HR-FAB-MS m/z found: 299.032 $C_{15}H_{11}N_2OS_2$ (M+H$^+$): requires M, 299.0313.

4.3.5. General procedure for the preparation of the benzothiazolium salts 5 and 14a,b. The appropriate benzothiazole (1 mmol) was heated with methyl tosylate (0.56 g, 3 mmol) at 100 °C for 16 h. The glassy solid formed was dissolved in DMF (2 mL) and water (15 mL) was added. The mixture was refrigerated over night and the crystals formed were collected by filtration.

4.3.5.1. 6-(6-methyl-benzothiazol-2-yl)-3-methyl-2-thio methyl-benzothiazolium tosylate 5. Obtained from the benzothiazole **4** (0.33 g, 1 mmol) using the above-described procedure (0.21 g, 40%). ^{1}H NMR (methanol-d_4): δ 2.35 (3H, s, Ar-CH_3), 2.53 (3H, s, Ar-CH_3), 3.18 (3H, s, S-CH_3), 4.18 (3H, s, NCH_3), 7.21 (1H, d, $J = 8$, ArH), 7.45 (2H, d, $J = 8$, ArH), 7.69 (1H, d, $J = 8$, ArH), 7.78 (2H, d, $J = 8$, ArH), 7.87 (1H, s, ArH), 7.96 (1H, d, $J = 8$, ArH), 8.20 (1H, d, $J = 8$, ArH), 8.92 (1H, s, ArH); HR-FAB-MS m/z found: 343.050 $C_{17}H_{15}N_2S_3$ (M$^+$): requires M, 343.0397.

4.3.5.2. 6-(Benzothiazol-2-yl)-3-methyl-2-thiomethyl-benzothiazolium tosylate 14a. Obtained from the benzothiazole **13a** (0.31 g, 1 mmol) using the above-described procedure (0.19 g, 37%). ^{1}H NMR (DMSO-d_6): δ 2.28 (3H, s, Ar-CH_3), 3.17 (3H, s, S-CH_3), 4.15 (3H, s, NCH_3), 7.10 (2H, d, $J = 8$, ArH), 7.46 (2H, d, $J = 8$, ArH), 7.54 (1H, t, $J = 8$, ArH), 7.62 (1H, t, $J = 8$, ArH), 8.13 (1H, d, $J = 8$, ArH), 8.24 (1H, d, $J = 8$, ArH), 8.35 (1H, d, $J = 8$, ArH), 8.52 (1H, d, $J = 8$, ArH), 9.20 (1H, s, ArH); HR-FAB-MS m/z found: 329.032 $C_{16}H_{13}N_2S_3$ (M$^+$): requires M, 329.0241.

4.3.5.3. 6-(Benzoxazol-2-yl)-3-methyl-2-thiomethyl-benzothiazolium tosylate 14b. Obtained from the benzothiazole **13b** (0.30 g, 1 mmol) using the above-described procedure (0.21 g, 43%). ^{1}H NMR (DMSO-d_6): δ 2.28 (3H, s, Ar-CH_3), 3.18 (3H, s, S-CH_3), 4.15 (3H, s, NCH_3), 7.10 (2H, d, $J = 8$, ArH), 7.47 (4H, m, ArH), 7.88 (2H, m, ArH), 8.41 (1H, d, $J = 8$, ArH), 8.61 (1H, d, $J = 8$, ArH), 9.29 (1H, s, ArH); HR-FAB-MS m/z found: 313.0459 $C_{16}H_{13}N_2OS_2$ (M$^+$): requires M, 313.0469.

4.3.6. General procedure for the cyanine dye condensations. The 2-thiomethyl benzothiazolium salt **5**, **14a** or **14b** (0.05 mmol) and the appropriate picolinium or lepidinium salt (0.05 mmol) were stirred in dichloromethane (2 mL). Triethyl amine (0.2 mmol, 20 mg, 28 μL) was added to form a deeply colored mixture, which was stirred at rt for 20 h. The mixture was concentrated and the residue purified by flash chromatography on neutral Al_2O_3 with methanol–dichloromethane (2–4% MeOH) as eluant. During chromatography, the tosylate counterion was in all cases exchanged for chloride, as judged by absence of tosylate protons in the NMR spectra and positive silver halide tests. The chloride anions most likely originate from traces of HCl formed by decomposition of the chlorinated solvent. Chemical shifts in deuterated methanol were found to be quite sensitive to concentration, probably due to aggregation. The data obtained here are from solutions of roughly 3 mg mL^{-1}.

4.3.6.1. 4-[(3-Methyl-6-(6-methyl-benzothiazol-2-yl)-2,3,-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (BEBO). Obtained from the benzothiazolium salt **5** (26 mg, 0.05 mmol) and 4-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (10 mg, 46%). ^{1}H NMR (DMSO-d_6): 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, $J = 8$, ArH), 7.47 (2H, d, $J = 7$, PyH), 7.70 (1H, d, $J = 8$, ArH) 7.93 (1H, d, $J = 8$, ArH), 7.95 (1H, s, ArH), 8.18 (1H, d, $J = 8$, ArH), 8.39 (2H, d, $J = 7$, PyH), 8.65 (1H, s, ArH). These data were consistent with those of the previously prepared compound.[13]

4.3.6.2. 2-[(3-Methyl-6-(6-methyl-benzothiazol-2-yl)-2,3,-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (2-BEBO). Obtained from the benzothiazolium salt **5** (26 mg, 0.05 mmol) and 2-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (9.5 mg, 43%). ^{1}H NMR (methanol-d_4): δ 2.46 (3H, s, Ar-CH_3), 3.70 (3H, s, N-CH_3), 3.91 (3H, s, N-CH_3), 5.56 (1H, s, =CH—), 7.10 (1H, t, $J = 8$, ArH), 7.30 (1H, d, $J = 8$, ArH), 7.46 (1H, d, $J = 8$, ArH) 7.65 (1H, s, $J = 8$, ArH), 7.78 (2H, m, ArH), 8.03 (1H, t, $J = 8$, ArH), 8.07 (1H, d, $J = 8$, ArH), 8.23 (1H, d, $J = 7$, ArH) 8.26 (1H, s, ArH); HR-FAB-MS m/z found: 402.104 $C_{23}H_{20}N_3S_2$ (M$^+$): requires M, 402.1099; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 437 (33,500), 374 (11,500), 340 (10,400), 273 nm (11,000 M^{-1} cm^{-1}).

4.3.6.3. 4-[(3-Methyl-6-(6-methyl-benzothiazol-2-yl)-2,3,-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (Me-BETO). Obtained from the benzothiazolium salt **5** (26 mg, 0.05 mmol) and 4-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red solid (15 mg, 61%). ^{1}H NMR (methanol-d_4): δ 2.50 (3H, s, Ar-CH_3), 4.03 (3H, s, N-CH_3), 4.25 (3H, s, N-CH_3), 6.97 (1H, s, =CH—),

7.36 (1H, d, $J = 8$, ArH), 7.56 (1H, d, $J = 7$, ArH), 7.75–7.89 (4H, m, ArH) 8.0–8.10 (2H, m, ArH), 8.27 (1H, d, $J = 7$, ArH), 8.51 (1H, d, $J = 7$, ArH), 8.58 (1H, s, ArH), 8.70 (1H, d, $J = 8$, ArH); HR-FAB-MS m/z found: 452.130 $C_{27}H_{22}N_3S_2$ (M^+): requires M, 452.1255; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 489 (41,500), 356 (11,500), 292 nm (15,000 M^{-1} cm^{-1}).

4.3.6.4. 2-[(3-Methyl-6-(6-methyl-benzothiazol-2-yl)-2,3,-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (Me-2-BETO). Obtained from the benzothiazolium salt **5** (26 mg, 0.05 mmol) and 2-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red-brown solid (17 mg, 68%). ^{1}H NMR (DMSO-d_6): δ 2.495 (3H, s, Ar-CH_3), 3.96 (3H, s, N-CH_3), 4.18 (3H, s, N-CH_3), 6.24 (1H, s, =CH—), 7.39 (1H, d, $J = 8$, ArH), 7.67 (1H, t, $J = 8$, ArH), 7.88 (1H, d, $J = 8$, ArH) 7.96 (3H, m, ArH), 8.08 (1H, d, $J = 8$, ArH), 8.13 (1H, d, $J = 9$, ArH), 8.21 (1H, d, $J = 9$, ArH), 8.26 (1H, d, $J = 9$, ArH), 8.57 (1H, d, $J = 9$, ArH), 8.76 (1H, s, ArH); HR-FAB-MS m/z found: 452.137 $C_{27}H_{22}N_3S_2$ (M^+): requires M, 452.1255; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 459 (34,000), 349 nm (12,500 M^{-1} cm^{-1}).

4.3.6.5. 4-[6-(Benzothiazol-2-yl)-(3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (H-BEBO). Obtained from the benzothiazolium salt **14a** (25 mg, 0.05 mmol) and 4-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (10 mg, 47%). ^{1}H NMR (DMSO-d_6): 3.76 (3H, s, N-CH_3), 4.03 (3H, s, N-CH_3), 6.35 (1H, s, =CH—), 7.48 (3H, m, ArH), 7.57 (1H, t, $J = 8$, ArH), 7.71 (1H, d, $J = 8$, ArH), 8.05 (1H, d, $J = 8$, ArH), 8.17 (1H, d, $J = 8$, ArH), 8.21 (1H, d, $J = 8$, ArH), 8.40 (2H, d, $J = 7$, ArH), 8.69 (1H, s, ArH); HR-FAB-MS m/z found: 388.095 $C_{22}H_{18}N_3S_2$ (M^+): requires M, 388.0942; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 444 (32,500), 339 (9500), 277 nm (6000 M^{-1} cm^{-1}).

4.3.6.6. 2-[6-(Benzothiazol-2-yl)-(3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (H-2-BEBO). Obtained from the benzothiazolium salt **14a** (25 mg, 0.05 mmol) and 2-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (16.5 mg, 79%). %). ^{1}H NMR (methanol-d_4): 3.83 (3H, s, N-CH_3), 4.11 (3H, s, N-CH_3), 5.89 (1H, s, =CH—), 7.24 (1H, t, $J = 7$, ArH), 7.45 (1H, t, $J = 7$, ArH), 7.55 (1H, d, $J = 8$, ArH), 7.63 (1H, d, $J = 8$, ArH), 8.0–8.06 (3H, m, ArH), 8.15 (1H, t, $J = 7$, ArH), 8.23 (1H, d, $J = 8$, ArH), 8.41 (1H, d, $J = 7$ ArH), 8.50 (1H, s, ArH); HR-FAB-MS m/z found: 388.094 $C_{22}H_{18}N_3S_2$ (M^+): requires M, 388.0942; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 437 (37,000), 371 (10,500), 275 nm (12,500 M^{-1} cm^{-1}).

4.3.6.7. 4-[6-(Benzothiazol-2-yl)-(3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (BETO). Obtained from the benzothiazolium salt **14a** (25 mg, 0.05 mmol) and 4-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red solid (19 mg, 79%). ^{1}H NMR (methanol-d_4): 4.04 (3H, s, N-CH_3), 4.25 (3H, s, N-CH_3), 6.98 (1H, s, =CH—), 7.44 (1H, t, $J = 8$, ArH), 7.52–7.58 (2H, m, ArH), 7.77 (1H, d, $J = 8$, ArH), 7.83 (1H, t, $J = 8$, ArH), 7.99–8.10 (4H, m, ArH), 8.30 (1H, dd, $J_1 = 8$, $J_2 = 2$, ArH), 8.51 (1H, d, $J = 7$, ArH), 8.62 (1H, s, ArH), 8.71 (1H, d, $J = 8$, ArH). These data were consistent with those of the previously prepared compound.[14] However, the shifts are somewhat different, which is explained by a lower dye concentration in the present NMR sample compared to the previously reported data (vide supra).

4.3.6.8. 2-[6-(Benzothiazol-2-yl)-(3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (2-BETO). Obtained from the benzothiazolium salt **14a** (25 mg, 0.05 mmol) and 2-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red-brown solid (21 mg, 89%). ^{1}H NMR (methanol-d_4): 3.98 (3H, s, N-CH_3), 4.23 (3H, s, N-CH_3), 6.22 (1H, s, =CH—), 7.45 (1H, t, $J = 8$, ArH), 7.55 (1H, t, $J = 8$, ArH), 7.67 (1H, t, $J = 8$, ArH), 7.78 (1H, d, $J = 8$, ArH), 7.96 (1H, t, $J = 7$, ArH), 7.99-8.05 (3H, m, ArH), 8.13 (1H, d, $J = 9$, ArH), 8.22 (1H, d, $J = 9$, ArH), 8.31 (1H, dd, $J_1 = 8$, $J_2 = 2$, ArH), 8.43 (1H, d, $J = 9$, ArH), 8.62 (1H, s, ArH); HR-FAB-MS m/z found: 438.115 $C_{26}H_{20}N_3S_2$ (M^+): requires M, 438.1099; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 468 (37,500), 355 (13,000), 279 nm (13,000 M^{-1} cm^{-1}).

4.3.6.9. 4-[6-(Benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (BOXBO). Obtained from the benzothiazolium salt **14b** (24 mg, 0.05 mmol) and 4-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (8 mg, 40%). ^{1}H NMR (methanol-d_4): 3.75 (3H, s, N-CH_3), 4.03 (3H, s, N-CH_3), 6.17 (1H, s, =CH—), 7.40–7.43 (5H, m, ArH), 7.59 (1H, d, $J = 8$, ArH), 7.64–7.71 (2H, m, ArH), 8.15 (1H, d, $J = 7$, ArH), 8.31 (1H, d, $J = 8$, ArH), 8.54 (1H, s, ArH); HR-FAB-MS m/z found: 372.112 $C_{22}H_{18}N_3OS$ (M^+): requires M, 372.1171; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 440 (36,500), 329 (6400), 271 nm (11,000 M^{-1} cm^{-1}).

4.3.6.10. 2-[6-(Benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium chloride (2-BOXBO). Obtained from the benzothiazolium salt **14b** (24 mg, 0.05 mmol) and 2-picolinium tosylate (14 mg, 0.05 mmol) using the above-described procedure as a yellow solid (16.5 mg, 81%). ^{1}H NMR (DMSO-d_6): 3.82 (3H, s, N-CH_3), 4.12 (3H, s, N-CH_3), 5.94 (1H, s, =CH—), 7.37 (1H, t, $J = 8$, ArH), 7.44 (2H, m, ArH), 7.76–7.83 (3H, m, ArH), 7.97 (1H, d, $J = 8$, ArH), 8.24–8.32 (2H, m, ArH), 8.62 (1H, d, $J = 7$, ArH), 8.74 (1H, s, ArH); HR-FAB-MS m/z found: 372.107 $C_{22}H_{18}N_3OS$ (M^+): requires M, 372.1171; UV–vis (sodium phosphate buffer 5 mM,

pH 7.0): λ_{max} (ε) = 436 (41,000), 362 (12,000), 323 (11,000), 270 nm (16,000 M^{-1} cm^{-1}).

4.3.6.11. 4-[6-(Benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (BOXTO). Obtained from the benzothiazolium salt **14b** (24 mg, 0.05 mmol) and 4-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red solid (12 mg, 53%). ^{1}H NMR (methanol-d_4): 4.05 (3H, s, N-CH_3), 4.28 (3H, s, N-CH_3), 7.02 (1H, s, =CH—), 7.40–7.44 (2H, m, ArH), 7.61 (1H, d, $J = 6$, ArH), 7.66–7.77 (2H, m, ArH), 7.81–7.87 (2H, m, ArH), 8.05 (1H, t, $J = 8$, ArH), 8.12 (1H, d, $J = 8$, ArH), 8.45 (1H, dd, $J_1 = 8$, $J_2 = 2$, ArH), 8.55 (1H, d, $J = 8$, ArH), 8.73 (1H, s, ArH), 8.74 (1H, d, $J = 8$, ArH). These data were consistent with those of the previously prepared compound.[14] However, the shifts are somewhat different, which is explained by a lower dye concentration in the present NMR sample compared to the previously reported data (vide supra).

4.3.6.12. 2-[6-(Benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride (2-BOXTO). Obtained from the benzothiazolium salt **14b** (24 mg, 0.05 mmol) and 2-lepidinium tosylate (16 mg, 0.05 mmol) using the above-described procedure as a red-brown solid (17 mg, 76%). ^{1}H NMR (methanol-d_4): 3.99 (3H, s, N-CH_3), 4.24 (3H, s, N-CH_3), 6.24 (1H, s, =CH—), 7.40–7.47 (2H, m, ArH), 7.66–7.79 (3H, m, ArH), 7.82 (1H, d, $J = 8$, ArH), 7.97 (1H, t, $J = 8$, ArH), 8.03 (1H, d, $J = 8$, ArH), 8.15 (1H, d, $J = 9$, ArH), 8.24 (1H, d, $J = 9$, ArH), 8.41-8.49 (2H, m, ArH), 8.70 (1H, s, ArH); HR-FAB-MS m/z found: 422.127 $C_{26}H_{20}N_3OS$ (M^+): requires M, 422.1327; UV–vis (sodium phosphate buffer 5 mM, pH 7.0): λ_{max} (ε) = 463 (37,500), 344 (13,500), 274 nm (15,500 M^{-1} cm^{-1}).

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