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New Sensitive Fluorophores for Selective DNA Detection

Brenno A. D. Neto,*,† Alexandre A. M. Lapis,‡ Fabiana S. Mancilha,‡ Igor B. Vasconcelos,† Caroline Thum,† Luiz A. Basso,† Diógenes S. Santos,† and Jaïrton Dupont*,‡

Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Tecnopuc, PUC-RS, Brazil, and Institute of Chemistry, UFRGS Porto Alegre, RS 90501-970, Brazil

brenno.ipi@gmail.com; dupont@iq.ufrgs.br

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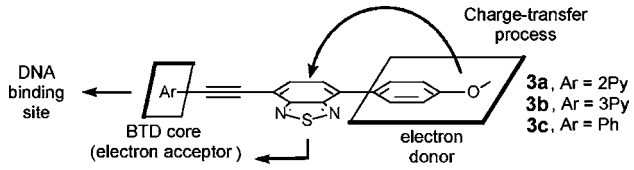
ABSTRACT



4,7-Disubstituted benzothiadiazoles containing 1-arylethynyl and 4-methoxyphenyl groups are selective photoluminescent "light up" probes to duplex DNA with unprecedented sensibility in both spectrophotometric and spectrofluorimetric measurements.

Biosensor technologies that focus on the direct detection of nucleic acids are currently an area of tremendous interest as they play a major role in clinical, forensic, and pharmaceutical applications.[1,2] The molecular probes that cause an increase in both absorbance and emission intensity ("light up" probes) by association with the host biomacromolecules (e.g., DNA, RNA, and proteins) are very useful photoluminescent markers in genomics and proteomics.[3] These simple and straightforward spectroscopic methods are especially advantageous because small organic dyes absorb and emit at wavelengths that do not interfere with the absorption of the DNA bases (λ max $\approx$ 260 nm). Indeed, spectrophotometric and spectrofluorimetric titrations are direct methodologies that indicate the association of a specific dye with DNA.[4]

The ability of planar polycyclic aromatic molecules to intercalate, i.e., to be inserted between two consecutive base pairs of DNA, is the basic molecular geometry of many intercalators that are used in antitumor chemotherapy,[5] as DNA cleavage agents[6] and fluorescent DNA intercalators,[7] and for various other purposes.[8] However, in the case of small molecular organic fluorophores, this association is not very clear, mainly because of the great diversity of the possible resulting structures.[9] Cationic organic dyes normally enhance the propensity of a small probe to bind to DNA, most via an interaction of the positive charge with the phosphate backbone in the double-strand DNA macromolecules.[10,11] However, these charged dyes have some draw-

* To whom correspondence should be addressed. Phone: 55 51 3320 3629 and 55 51 3308 6321.
† CPBMF.
‡ UFRGS Porto Alegre.

(1) (a) Wang, J. *Nucleic Acids Res.* **2000**, *28*, 3011−3016. (b) Niemeyer, C. M.; Blohm, D. *Angew. Chem., Int. Ed.* **1999**, *38*, 2865−2869.
(2) (a) Pu, L. *Chem. Rev.* **2004**, *104*, 1687−1716. (b) Valeur, B. *Molecular Fluorescence: Principles and Applications*; Wiley-VCH: Weinheim, 2002.
(3) Prentø, P. *Biotech. Histochem.* **2001**, *76*, 137−161.
(4) Cantor, C. R.; Schimmel, P. R. *In Biophysical Chemistry*; W. H. Freeman and Co.: San Francisco, 1980; Part II, pp 392−463.
(5) (a) Denny, W. A.; Baguley, B. In *Molecular Aspects of Anti-Cancer Drug-DNA Interaction*; Neidle, S., Warning, M., Eds.; Macmillan: London 1994. (b) Wang, L.; Price, H. L.; Juusola, J.; Kline, M.; Phanstiel, O., IV *J. Med. Chem.* **2001**, *44*, 3682−3691. (c) Lee, Y.-A.; Lee, S.; Cho, T.-S.; Kim, C.; Han, S. W.; Kin, S. K. *J. Phys. Chem. B* **2002**, *106*, 11351−11355.
(6) Viola, G.; Acqua, F. D.; Gabellini, N.; Moro, S.; Vedaldi, D.; Ihmels, H. *ChemBioChem* **2002**, *3*, 550−558.
(7) For a review, see: Erkkila, K. E.; Odom, D. T.; Barton, J. K. *Chem. Rev.* **1999**, *99*, 2777−2795.
(8) Cholody, W. M.; Kosakowka-Cholody, T.; Hollingshead, M. G.; Hairprakasha, H. K.; Michejda, C. J. *J. Med. Chem.* **2005**, *48*, 4474−4481.
(9) Granzhan, A.; Ihmels, H.; Viola, G. *J. Am. Chem. Soc.* **2007**, *129*, 1254−1267.

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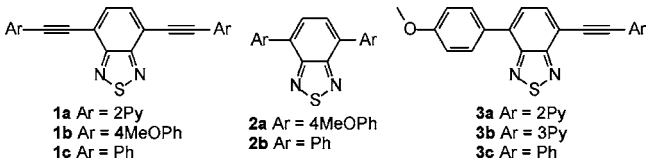
backs especially in electrophoresis purification, due to the concomitant migration of the cation and the anion along the gel. Therefore, the DNA bands in the agarose gel sometimes get poorly resolved as in the case of the commercially available ethidium bromide, which is widely used for DNA visualization in agarose gel electrophoresis.[12] Furthermore, the design of new small molecular organic fluorophores is not a simple task.[9] Higher selectivity, sensitivity, shorter assay times, and greater simplicity in performing the assay are trends that must be taken into account in the design of new intercalators that may be commercially viable.[13]

Neutral and highly polar dyes as DNA intercalators are rare examples,[14] but they can be also used for visualization of biomacromolecules. In this respect, fluorophores such as quinoxalines,[15] benzimidazoles,[16] and 2,1,3-benzothiadiazoles[17] have widespread use in scientific and technological areas,[18] mainly in light technology applications.[19] In this respect, we have recently shown that the photophysical properties of 2,1,3-benzothiadiazoles (BTD) can be tuned by proper choice of the nature of the π-extension in positions 4 and 7.[20−22]

These molecules possess the geometry and electronic properties desirable for DNA duplex intercalators. Here, we demonstrate that new neutral and highly polar BTD are outstanding light up probes for human DNA.

In order to check both electronic and geometrical parameters on the properties of BTD as light up probes to DNA, we have changed the groups attached to the 4,7-BTD core (Chart 1).

Chart 1. Tested BTD Dyes **1a**−**c**, **2a**,**b**, and **3a**−**c**



The dyes **1a**−**c** and **2a,b** were synthesized using a methodology previously described.[20,22] The BTD **3a**−**c** were synthesized combining both Sonogashira and Suzuki coupling reactions (see the Supporting Information for details).

The photophysical properties of compounds **1a**−**c**, **2a**,**b**, and **3a**−**c** were performed in phosphate buffer solutions (100 mM, pH = 7.0), and the results obtained are summarized in Table 1.

Table 1. UV−vis and Fluorescence of the BTD Dyes **1a**−**c**, **2a**,**b**, and **3a**−**c** in Phosphate Buffer 100 mM (pH = 7.0)

dye	log ϵ (ϵ)	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	Stokes shift (nm)	Φ_f [a]
1a	4.35 (22342)	365	471	106	0.86
1b	3.76 (5790)	429	563	134	0.29
1c	3.81 (6482)	438	552	114	0.37
2a	3.22 (1644)	411	535	124	0.51
2b	3.33 (2150)	367	506	139	0.80
3a	3.97 (9152)	444	544	100	0.40
3b	3.78 (6076)	401	547	146	0.44
3c	4.01 (10208)	426	525	99	0.47

[a] Quantum yield of fluorescence [quinine sulfate in 1 M H_2SO_4, f = 0.55, as standard].

In phosphate buffer (100 mM, pH = 7.0), the lowest energy absorption bands for compounds **1a**−**c**, **2a**,**b**, and **3a**−**c** (see the Supporting Information) are assigned to $\pi-\pi^*$ transitions by virtue of their large molar extinction coefficients (log ϵ values in the range of 3.22−4.35). The absorption (λ_{abs}^{max}) and emission (λ_{em}^{max}) maxima lie between 365 and 444 nm and 471−563 nm, respectively. It is worth noting that all dyes (**1a**−**c**, **2a**,**b**, and **3a**−**c**) have large Stokes shifts in solution, 99−146 nm, allowing unambiguous detection without reabsorption effects and not interfering with the background fluorescence of biomolecules. These high values also indicate a very efficient intramolecular charge transfer (ICT) in the excited state between the terminal aromatic group (phenyl ring or a methoxyphenyl group) and the BTD moiety.

Spectrophotometric Titrations. All synthesized dyes showed λ_{abs}^{max} in the near-UV region of the spectrum (~390 nm), well separated from the one of the nucleic bases (~260 nm). Negative results were obtained during titration using the systems **2a,b** since it is necessary to use a high concentration of the intercalating agent and high concentrations of DNA. However, all compounds having a C≡C π spacer **1a**−**c** and especially **3a**−**c**, were successfully tested as sensitive probes for selective DNA detection (Figure 1(A) and Figures S4 and S6, Supporting Information). This fact indicated the necessity of the triple bound spacer C≡C in order to facilitate the intercalation binding between the dyes and DNA duplex. All compounds (**1a**−**c**, **2a**,**b**, and **3a**−**c**) were also tested against human and *Mycobacterium tuberculosis* purine nucleoside phosphorylase (PNP) enzymes with nega-

(10) Medhi, C.; Mitchell, J. B. O.; Price, S. L.; Tabor, A. B. *Biopolymers* **1999**, *52*, 84−93.
(11) Ihmels, H.; Faulhaber, K.; Vedaldi, D.; Dall'Acqua, F.; Viola, G. *Photochem. Photobiol.* **2005**, *81*, 1107−1115.
(12) (a) Luedtke, N.; Liu, Q.; Tor, Y. *Chem. Eur. J.* **2005**, *11*, 495−508. (b) Heller, D. P.; Greenstock, C. L. *Biophys. Chem.* **1994**, *50*, 305.
(13) Wong, E. L. S.; Gooding, J. *J. Anal. Chem.* **2006**, *78*, 2138.
(14) For example, see: Valis, L.; Enthart, E. M.; Wagenknecht, H-A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3184−3187.
(15) Cui, Y.; Zhang, X.; Jenekhe, S. A. *Macromolecules* **1999**, *32*, 3824−3826.
(16) Gao, Z. Q.; Lee, C. S.; Bello, I.; Lee, S. T.; Wu, S. K.; Yan, Z. L.; Zhang, X. H. *Synth. Met.* **1999**, *105*, 141−144.
(17) (a) Akhtaruzzaman, M.; Tomura, M.; Nishida, J.; Yamashita, Y. *J. Org. Chem.* **2004**, *69*, 2953−2958. (b) Akhtaruzzaman, M.; Tomura, M.; Zaman, M. B.; Nishida, J.; Yamashita, Y. *J. Org. Chem.* **2002**, *67*, 7813−7818.
(18) Lee, M. T.; Yen, C. K.; Yang, W. P.; Chen, H. H.; Liao, C. H.; Tsai, C. H.; Chen, C. H. *Org. Lett.* **2004**, *6*, 1241−1244.
(19) Kraft, A.; Grimsdale, C. A.; Homes, B. A. *Angew. Chem., Int. Ed.* **1998**, *37*, 402−428.
(20) Mancilha, F. S.; Neto, B. A. D.; Lopes, A. S.; Moreira, P. F., Jr.; Quina, F. H.; Gonçalves, R. S.; Dupont, J. *Eur. J. Org. Chem.* **2006**, 4924−4933.
(21) Neto, B. A. D.; Lopes, A. S.; Wust, M.; Costa, V. E. U.; Ebeling, G.; Dupont, J. *Tetrahedron Lett.* **2005**, *46*, 6843−6846.
(22) Neto, B. A. D.; Lopes, A. S.; Ebeling, G.; Goncalves, R. S.; Costa, V. E. U.; Quina, F. H.; Dupont, J. *Tetrahedron* **2005**, *61*, 10975−10982.

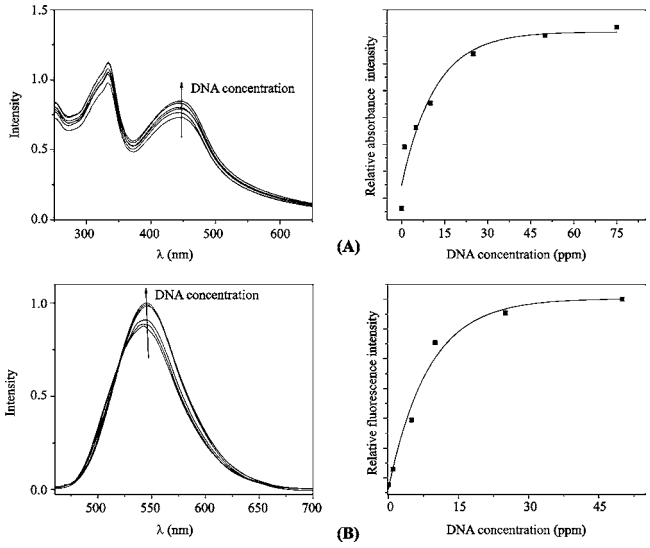


Figure 1. Spectrophotometric (**A**) and Spectrofluorimetric (**B**) titrations of DNA to compound **3a** (10 μM in a phosphate buffer (100 mM) in all measurements) (ppm = ng/mL).

tive results, indicating their sensitivity and selectivity toward DNA.

The addition of DNA (10−100 ppm) to the buffered solutions of the BTD **1a** (50 μM) causes a significant decrease of the absorbance (hypochromic effect) and a blue shift (3−5 nm) of the long wavelength absorption maxima. The exponential decay of the relative absorbance intensity furnishes a quantitative detection of the DNA using BTD **1a**. Note that using spectrophotometric titration, this compound is the only one that acts as a "light off" probe, and this clearly indicates the importance of a donor group (PhOMe) attached to the BTD core.

Compound **1b** (50 μM) is not a good candidate due to the pronounced rotational bands that result in a poorly resolved spectra. Moreover, it indicates that the interaction occurs, increasing the absorbance intensity exponentially upon binding with DNA, and the correlation with the biomacromolecules is almost perfect. Among compounds **1a**−**c**, BTD **1c** showed the best results using spectrophotometric titration. Upon addition of DNA (5−75 ppm) to the buffered solutions of the BTD **1c** (50 μM), a significant increase of the absorbance (hyperchromic effect) and a red shift (5−12 nm) of the long wavelength absorption maxima was observed.

The dyes **3a**−**c** gave the best results among all compounds (Figure 1(A) and Figures S4 and S6). The molecular architecture designed for these sensitive dyes indicates the efficiency of our strategy of combining in the organic systems a donating group (4-MeOPh) directly attached to the BTD nucleus on one side and the presence of C≡C π spacer on the other.

Compounds **3a**−**c** possess high sensitivity to DNA detection, i.e., down to 1 ppm of DNA (the hyperchromic effect observed were very pronounced, especially observed for compound **3c)**. A red shift between 2 and 7 nm of the long wavelength absorption maxima was observed for compound **3a**, 2−5 nm for **3b**, and impressive 5−19 nm for BTD **3c**. To the best of our knowledge, these series (**3a**−**c**) are among the most sensitive light up probes for DNA detection using spectrophotometric titrations reported to date.

Spectrofluorimetric Titrations. Spectrofluorimetric titrations of DNA to the BTD derivatives **1a**−**c**, **2a**,**b**, and **3a**−**c** were performed in an aqueous buffer solution at a ligand concentration of 10 μM. Under these conditions, no precipitation was observed. No significant results were obtained using the BTD systems **2a**,**b**, which indicates that even in a more sensitive technical analysis (fluorescence), the presence of a C≡C π spacer is fundamental to allow the binding of the dye to the DNA duplex. All compounds (**1a**−**c**, **2a**,**b**, and **3a**−**c**) were equally tested against human and *M. tuberculosis* purine nucleoside phosphorylase (PNP) enzymes with negative results indicating once more their sensitivity and selectivity to DNA.

Compounds **1a**−**c** showed interesting results (see Figure S6 in the Supporting Information). In particular, **1b** presents a very different behavior than all other BTD. This compound decomposes upon irradiation at 429 nm. Compounds **1a** and **1c**, however, apparently could be useful as light up probes to DNA detection using fluorimetric titration. In both cases, the dyes used in very low concentration (10 μM) could detect even 1 ppm of DNA in phosphate buffer solutions. Nevertheless, after a few weeks in the stock solution, we observe degradation of compound **1c.** Compound **1a** presented its long wavelength emission maximum below 500 nm (473 nm for **1a**, see Table 1) and therefore not suitable for the detection of fluorescence without distortion by autofluorescence of the cell matrix.[23] However, dyes **3a**−**c** present the best results for fluorescence detection and quantification (Figure 1 (B) and Figures S4 and S6). The insertion of one 4-MeOPh group on the molecular architecture of compounds **3a**−**c** increases the thermal, electrochemical, and excited-state stability of the small fluorescent organic systems.

In all cases, the dyes used in very low concentration (10 μM) could detect even 1 ppm of DNA in phosphate buffer solutions. BTD **3a** showed a significant increase of the fluorescence intensity (hyperchromic effect) and a red shift (2−5 nm) of the long-wavelength emission maximum. The exponential increase of the relative fluorescence intensity enabled us to carry out a quantitative detection of the DNA using BTD **3a** in an almost perfect match as seen in Figure 1.

Compounds **3b** and **3c** presented similar results with a significant increase in the fluorescence intensity and a slight red shift (1−4 nm and 2−3 nm, respectively) of the long wavelength emission maxima. Both fluorescent systems also presented an exponential increase of fluorescence upon increasing the DNA concentration. It is interesting for a comparison that commonly used florescent probes normally possess a detection limit that is equal to or above 10 ppm[24] (see Table T1 in the Supporting Information) such as the widely used ethidium bromide.[24a] In fact, BTD **3a**−**c**

(23) Patonay, G.; Antoine, M. D. *Anal. Chem.* **1991**, *63*, 321A-327A.

fluorescent light up probes, especially, are among the most sensitive probes ever described.

Upon binding with DNA, compounds **3a**−**c** maintain their high Stokes shift, with a red shift after the association with DNA, indicating a more efficient ICT process. If the association proceeded through the electron-donating group, the ICT process would not be expected to be so efficient. All results indicate that the intercalation occurs at the C≡C side of the BTD, and as a consequence, the PhOMe portion would be free to perform the ICT process with the BTD core, as rationalized in Figure 2. Preliminary stopped-flow kinetic

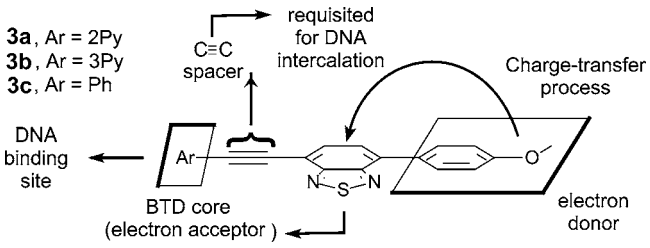


Figure 2. Proposed architecture upon DNA binding.

experiments indicate high binding constants (immediately binding to DNA).

A comparative and qualitative experiment of resolution and sensitivity to DNA was performed with dye **3a**, comparing with the commercially available ethidium bromide (Figure 3). The commercially available DNA plasmid (pCINeo) at different concentrations (200, 100, 50, 20, 10, 5 ng) was submitted in two different agarose gels (1%) to electrophoresis purification using commercial 1 kb plus as a control. It is clear from Figure 3 that the agarose gel A (used with compound **3a**) reveals DNA in the six different concentrations, while agarose gel B (used with ethidium bromide) reveals only the most concentrated DNA channel (200 ng). It is also worth noting that there is no migration of compound **3a**, as commonly occurs using charged dyes such as ethidium bromide. We can also note that using compound **3a**, we were able to reveal the bands with small mass (see the control band in Figure 3), while it is not possible with ethidium bromide (see Supporting Information for **3b**).

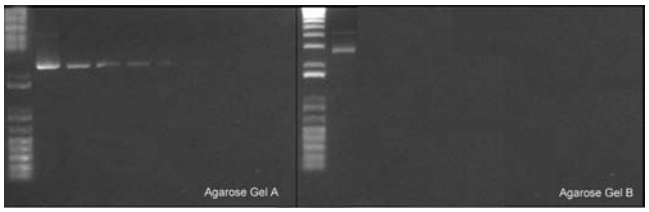


Figure 3. UV irradiation at 360 nm: (A) dye **3a** (1 μL of 1 mM solution) and (B) ethidium bromide (1 μL of 1 mM solution). Gel channels, left to right: control, 1 Kb plus; pCINeo, 200 ng; pCINeo, 100 ng; pCINeo, 50 ng; pCINeo, 20 ng; pCINeo, 10 ng; pCINeo, 5 ng.

In summary, BTDs containing a PhOMe donating group on one side associated with an Ar−C≡C group is a suitable molecular architecture for the spectroscopic selective detection and quantification of DNA. The new dyes are among the most sensitive probes for the DNA detection in solution by spectrophotometric and spectrofluorimetric titrations. A full intercalating model study with completely kinetic and binding model with stopped-flow, surface plasmon resonance, real time PCR, electrochemical, and theoretical approach will be published in due course.

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Supporting Information Available: Synthetic and titration experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) (a) Paigen, K. *Anal. Biochem.* **1980**, *102*, 344−352. (b) Li, W. Y.; Xu, J. G.; Guo, X. Q.; Zhu, Q. Z.; Zhao, Y. B. *Anal. Lett.* **1997**, *30*, 527−536. (c) Li, W. Y.; Xu, J. G., Zhu, Q.; Zhao, Y. *Anal. Lett.* **1997**, *30*, 245−257. (d) Sueda, S.; Ihara, T.; Juskowiak, B.; Takagi, M. *Anal. Chim. Acta* **1998**, *365*, 27−34. (e) Yang, J.; Tong, C.; Jie, N.; Gong, H. *Chin. Biochem. J.* **1996**, *12*, 143. (f) Ci, Y.; Li, Y. Z.; Chang, W. B. *Anal. Chim. Acta* **1991**, *248*, 589−594. (g) Ci, Y. X.; Li, Y. Z.; Liu, X. *J. Anal. Chem.* **1995**, *67*, 1785−1788. (h) Huang, C. Z.; Li, K. A.; Tong, S. Y. *Anal. Lett.* **1996**, *29*, 1705−1717. (i) Huang, C. Z.; Li, K. F.; Tong, S. Y. *Anal. Lett.* **1997**, *30*, 1305−1319. (j) Wu, X.; Yang, J.; Huang, F. *Anal. Lett.* **1999**, *32*, 2417−2425. (l) Liu, R. T.; Yang, J. H.; Wu, X. *J. Lumin.* **2002**, *96*, 201−209. (m) Skripinets, Y. V.; Egorova, A. V.; Ukrainets, I. V.; Antonovich, V. P. *J. Anal. Chem.* **2006**, *61*, 44−51. (n) LePecq, J. B.; Paoletti, C. *Anal. Biochem.* **1966**, *17*, 100.