ORGANIC LETTERS

2010
Vol. 12, No. 2
216−219

Bis-4-aminobenzamidines: Versatile, Fluorogenic A/T-Selective dsDNA Binders§

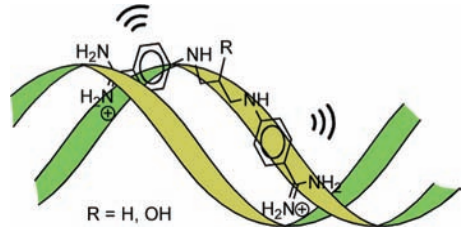
Olalla Vázquez,† Mateo I. Sánchez,† José Martínez-Costas,‡ M. Eugenio Vázquez,*,† and José L. Mascareñas*,†

Departamento de Química Orgánica and Departamento de Bioquímica y Biología Molecular, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

joseluis.mascarenas@usc.es

Received October 30, 2009

ABSTRACT



***N*¹,*N*⁸-Bis(4-amidinophenyl)propane-1,3-diamine (BAPPA, R = H) is a bisaminobenzamidine fluorogenic derivative that displays a large increase in its emission fluorescence when bound to the minor groove of specific A/T DNA sites (K_d for an AATT site ~ 79 ± 6 nM). Moreover, the structural characteristics of BAPPA allow the easy introduction of functional groups that protrude out of the DNA surface.**

Deciphering the human genome has opened new perspectives in biomedical research, promising improved diagnostic techniques and personalized therapies.[1] However, to fully exploit the knowledge about our genes, it is necessary to develop probes capable of targeting and sensing specific DNA sequences. While a number of sequence identification methods that rely on single-stranded hybridization have been successfully implemented, direct sensing of double-stranded DNA (dsDNA) without the need for denaturation is clearly underdeveloped.[2] Of the few strategies so far described, those based on distamycin-like hairpin polyamides are particularly remarkable.[3] However, adapting these systems for direct dsDNA sensing is not trivial, owing to synthetic requirements and cell internalization difficulties.[4] Therefore, new sequence-specific dsDNA probes based on simple, versatile, and readily accessible structural scaffolds are highly desirable.

Benzamidines such as pentamidine (**1**) and propamidine (**2**) (Figure 1) are very simple DNA binders that show good

§ Dedicated to Prof. Luis Castedo on the occasion of his 70th birthday.
† Departamento de Química Orgánica.
‡ Departamento de Bioquímica y Biología Molecular.

(1) (a) Shastry, B. S. *Pharmacogenomics J.* **2006**, *6*, 16–21. (b) Voelkerding, K. V.; Dames, S. A.; Durtschi, J. D. *Clin. Chem.* **2009**, *55*, 641–658.

(2) (a) Ghosh, I.; Stains, C. I.; Ooi, A. T.; Segal, D. J. *Mol. Biosyst.* **2006**, *2*, 551–560. (b) Chan, E. Y.; Goncalvez, N. M.; Haeusler, R. A.; Hatch, A. J.; Larson, J. W.; Maletta, A. M.; Yantz, G. R.; Carstea, E. D.; Fuchs, M.; Wong, G. G.; Gullans, S. R.; Gilmanshin, R. *Genome Res.* **2004**, *14*, 1137–1146. (c) Pianowski, Z. L.; Winssinger, N. *Chem. Soc. Rev.* **2008**, *37*, 1330–1336.

(3) Polyamides with appended dyes: (a) Rucker, V. C.; Foister, S.; Melander, C.; Dervan, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 1195–1202. (b) Fechter, E. J.; Olenyuk, B.; Dervan, P. B. *J. Am. Chem. Soc.* **2005**, *127*, 16685–16691. For a polyamide with an integrated fluorophore, see: (c) Chenoweth, D. M.; Viger, A.; Dervan, P. B. *J. Am. Chem. Soc.* **2007**, *129*, 2216–2217.

(4) (a) Best, T. P.; Edelson, B. S.; Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 12063–12068. (b) Vázquez, O.; Blanco-Canosa, J. B.; Vázquez, M. E.; Martínez-Costas, J.; Castedo, L.; Mascareñas, J. L. *ChemBioChem* **2008**, *9*, 2822–2829.

(5) (a) Tidwell, R. R.; Boykin, D. W. *DNA and RNA Binders: From Small Molecules to Drugs*; Demeunynck, M., Bailly, C., Wilson, W. D., Eds.; Wiley-VCH: Weinheim 2003; Vol. 2, p 414−460. (b) Lansiaux, A.; Tanious, F.; Mishal, Z.; Dassonneville, L.; Kumar, A.; Stephens, C. E.; Hu, Q.; Wilson, W. D.; Bokin, D. W.; Bailly, C. *Cancer Res.* **2002**, *62*, 7219–7229.

10.1021/ol902501j
Published on Web 12/10/2009

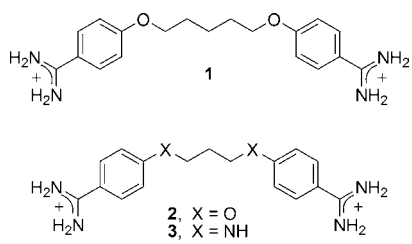


Figure 1. Pentamidine (**1**), propamidine (**2**), and BAPPA (**3**).

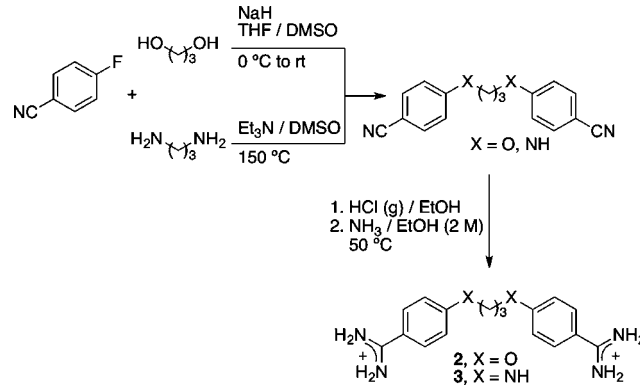
stability, A/T sequence selectivity, and excellent cell transport properties in a variety of cell lines.[5] Pentamidine and other derivatives are either in use or in clinical trials against several parasites like *P. vivax*, *P. carinii pneumonia*, or *P. falciparum malaria*,[6] and there is great interest in developing new analogues with improved efficacies and reduced toxicity. Curiously, most of the DNA-binding studies of such benzamidines are based on measurements of the thermal denaturation of their DNA complexes, a technique that only gives indirect and relatively coarse information of binding properties and is difficult to implement in high-throughput screenings.[7] Therefore, the development of simple and direct spectroscopic methods that allow a rapid monitoring and quantification of the DNA binding affinity and selectivity of these compounds would be highly desirable.[8]

Herein, we demonstrate that replacing the bis-4-amidinophenoxy unit found in classic benzamidine derivatives by a bis-4-aminobenzamidine yields propamidine analogues that display a large fluorescence enhancement upon sequence-specific dsDNA binding. This provides for a simple, rapid, and reliable method to monitor and quantify their DNA affinity and selectivity, as well as to study the binding of other nonfluorescent minor-groove binders.[8] Importantly, in contrast to classic nuclear stains such DAPI or Hoechst, these analogues can be very easily assembled, and their structure allows the straightforward introduction of functional groups oriented toward the outer side of the DNA minor groove.

This research aroused in the context of our search for a structurally simple and easily accessible substitute for the tripyrrole component used in previously developed bivalent minor−major groove DNA recognition systems.[9] We focused our attention on bisamidine systems owing to their structural simplicity and potential for installing appropriate handles for conjugation to the peptides.[10]

Although the electronic donor−acceptor configuration in the bis-4-amidinophenoxy units in **2** might provide for the observation of DNA-promoted fluorescence changes, the overlap between the excitation wavelength of **2** (260 nm) and the UV absorption of the DNA precludes its selective excitation in the presence of DNA. Therefore, we decided to prepare the *aza*-analogue of propamidine, namely, *N*[1],*N*[3]-*bis(4-amidinophenyl)propane-1,3-diamine* (BAPPA, **3**), featuring nitrogens instead of the two oxygen atoms present in the parent propamidine (**2**). We anticipated that the increased donating character of the amines would yield a push−pull system with longer excitation and emission wavelengths than **2**, together with a higher environment-sensitive fluorogenicity.[11] The synthesis of **2** and **3** was carried out in a straightforward manner using a slightly modified version of known procedures (Scheme 1).[12]

Scheme 1. Synthesis of Propamidine (**2**) and BAPPA (**3**)



As expected, propamidine analogue **3** (BAPPA) shows longer maximum excitation (329 nm) and emission (387 nm) wavelengths than propamidine (**2**). Moreover, the fluorescence emission intensity of BAPPA displays strong sensitivity to the solvent polarity, increasing from being almost nonfluorescent in water to displaying strong emission in MeOH or *i*PrOH. Interestingly, BAPPA displays weaker fluorescence in nonpolar solvents, and in some of them like

(6) (a) Wilson, W. D.; Tanious, F. A.; Mathis, A.; Tevis, D.; Hall, J. E.; Boykin, D. W. *Biochimie* **2008**, *90*, 999–1014. (b) Rodríguez, F.; Rozas, I.; Kaiser, M.; Brun, R.; Nguyen, B.; Wilson, W. D.; García, R. N.; Dardonville, C. *J. Med. Chem.* **2008**, *51*, 909–923. (c) Mayence, A.; Pietka, A.; Collins, M. S.; Cushion, M. T.; Tekwani, B. L.; Huang, T. L.; Eynde, J. J. V. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2658–2661.

(7) Wilson, W. D.; Tanious, F. A.; Fernandez-Saiz, M.; Rigl, C. T. *Methods Mol. Biol.* **1997**, *90*, 219–240.

(8) For examples of light-off fluorescence displacement assays for analyzing minor groove binding, see: (a) Tse, W. C.; Boger, D. L. *Acc. Chem. Res.* **2004**, *37*, 61–69. (b) Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. *J. Am. Chem. Soc.* **2001**, *123*, 5878–5891. (c) Jenkins, T. C. *Methods Mol. Biol.* **1997**, *90*, 195–218.

(9) (a) Vázquez, O.; Vázquez, M. E.; Blanco, J. B.; Castedo, L.; Mascareñas, J. L. *Angew. Chem., Int. Ed.* **2007**, *46*, 6886–6890. (b) Blanco, J. B.; Dodero, V. I.; Vázquez, M. E.; Mosquera, M.; Castedo, L.; Mascareñas, J. L. *Angew. Chem., Int. Ed.* **2006**, *45*, 8210–8214. (c) Blanco, J. B.; Vázquez, O.; Martínez-Costas, J.; Castedo, L.; Mascareñas, J. L. *Chem.—Eur. J.* **2005**, *11*, 4171–4178. (d) Blanco, J. B.; Vázquez, M. E.; Martínez-Costas, J.; Castedo, L.; Mascareñas, J. L. *Chem. Biol.* **2003**, *10*, 713–722. (e) Vázquez, M. E.; Caamaño, A. M.; Martínez-Costas, J.; Castedo, L.; Mascareñas, J. L. *Angew. Chem., Int. Ed.* **2001**, *40*, 4723–4725. (f) Blanco, J. B.; Vázquez, M. E.; Castedo, L.; Mascareñas, J. L. *ChemBioChem* **2005**, *6*, 2173–2177.

(10) For structural information on their DNA complexes, see: (a) Nunn, C. M.; Neidle, S. *J. Med. Chem.* **1995**, *38*, 2317–2325. (b) Nunn, C. M.; Jenkins, T. C.; Neidle, S. *Biochemistry* **1993**, *32*, 13838–13843. (c) Edwards, K. J.; Jenkins, T. C.; Neidle, S. *Biochemistry* **1992**, *31*, 7104–7109.

(11) For spectroscopic effects in push−pull conjugated systems: (a) Gáplovský, A.; Donovalová, J.; Magdolen, P.; Toma, Š.; Zahardník, P. *Spectrochim. Acta A* **2002**, *58*, 363–371. (b) Suzuki, K.; Demeter, A.; Kühnle, W.; Tauer, E.; Zachariasse, K. A.; Tobita, S.; Shizuka, H. *Phys. Chem. Chem. Phys.* **2000**, *2*, 981–991.

(12) (a) Tidwell, R. R.; Jones, S. K.; Geratz, J. D.; Ohemeng, K. A.; Cory, M.; Hall, J. E. *J. Med. Chem.* **1990**, *33*, 1252–1257. (b) Donkor, I. O.; Huang, T. L.; Tao, B.; Rattendi, D.; Lane, S.; Vargas, M.; Goldberg, B.; Bacchi, C. *J. Med. Chem.* **2003**, *46*, 1041–1048.

THF or 1,4-dioxane we also observed a dual emission profile (see the Supporting Information).[13]

More importantly, addition of a hairpin oligonucleotide containing the consensus binding region *AATTT* promoted a large fluorescence emission increase (>10-fold), probably as a consequence of the change in hydration and viscosity experienced by the probe when removed from the bulk water into the DNA minor groove.[14] In a titration experiment, addition of successive aliquots of such a hairpin oligonucleotide to a 0.5 μM solution of BAPPA in buffer led to the fluorescence profile represented in Figure 2. The increase in

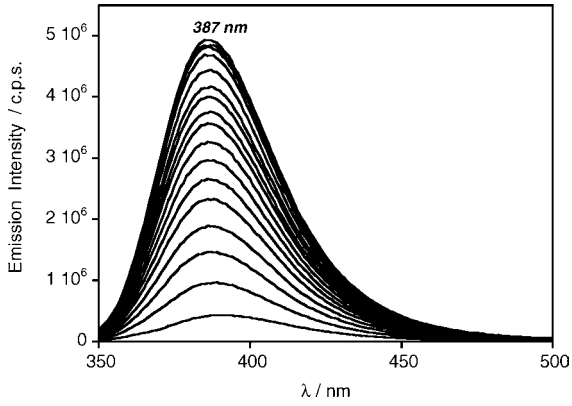


Figure 2. Titration of BAPPA (0.5 μM in Tris-HCl buffer 20 mM, pH 7.5, 100 mM NaCl) with the hairpin oligonucleotide 5′-GGCG *AATTT* CGC TTTTT GCG *AAATT* CGCC-3′.

the emission intensity at 387 nm could be fitted to a simple 1:1 binding mode.[15] The observed K_D of ~70 nM is in agreement with previously reported binding constants for propamidine and related minor groove binders.[16]

Likewise, titrations of BAPPA with different hairpin oligonucleotides with other sequences (Figure 3) allowed us to establish the binding site profile (Table 1). As expected, the preferred sequences were those containing at least four A/T base pairs, with *AATT* being slightly better than *AAAA*. Introduction of a single G/C mutation in the sequence led to a 15-fold decrease in affinity ($K_D \sim 1.1 \pm 0.3$ μM). Further mutations of the preferred A/T-rich site drastically reduce the binding affinity, with G/C-rich sequences showing only residual binding to BAPPA.

Curiously, when using a dsDNA featuring a longer binding site with 6 base pairs in length (*AAATTT*), the binding isotherm was better represented assuming that the dsDNA could accommodate two BAPPA molecules in the *AAATTT* site. This result recalls a recent report that describes simultaneous binding of other bisamidines in overlapping

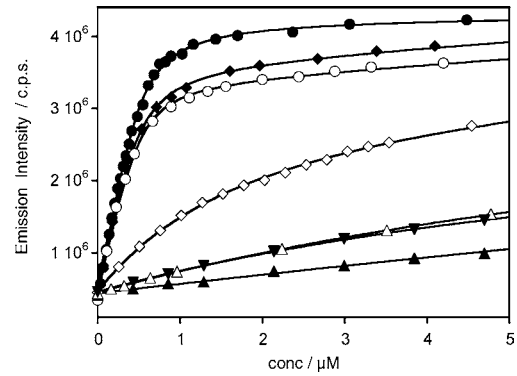


Figure 3. Titrations of BAPPA with selected DNA hairpin oligonucleotides containing the sequences: *AATTT* (●); *AATT* (○); *AAAA* (♦); *AATG* (◇); *AAGG* (▼); *ATGG* (Δ); and *GGCC* (▲). Binding curves represent the best fit to a 1:1 binding mode considering the contribution of the dsDNA to the fluorescence emission. Emission monitored at 387 nm.

sites in the minor groove, although in such cases the target sequences incorporate GC base pairs.[17]

Table 1. Sequence Selectivity of BAPPA from Titrations with Selected dsDNAs[a]

	dsDNA site[b]	K_D[c]
BAPPA	*GGCC*	~16 μM[d]
	ATGG	6.6 ± 0.5 μM
	AAGG	5.7 ± 1.3 μM
	AATG	1.1 ± 0.3 μM
	AAAA	85 ± 12 nM
	AATT	79 ± 6 nM
	AATTT	70 ± 10 nM

[a] All titrations were performed by addition of successive aliquots of the DNA stock solutions to a 0.5 μM solution of BAPPA (20 mM Tris-HCl buffer, 100 mM NaCl, pH 7.5). [b] See Supporting Information for full hairpin oligonucleotide sequences. [c] K_D values are the average of at least three independent titrations for each oligonucleotide DNA. [d] Saturation was not reached with this DNA even at high concentrations.

The structural simplicity and synthetic accessibility of BAPPA allows a straightforward preparation of analogues featuring functional group handles in the propyl tether. Therefore, the BAPPA derivative **4**, which incorporates the hydroxy group in the middle carbon, was very easily prepared as shown in Scheme 2. Binding assays revealed a slight loss in affinity with respect to BAPPA, for the consensus *AATTT* site ($K_D \sim 220$ nM, see the Supporting Information).

The BAPPA dsDNA-induced fluorescence can also be used to study the DNA binding profile of other A/T selective, nonfluorescent, minor groove binders, by means of competitive displacements. In a typical experiment, a solution of BAPPA and the hairpin oligonucleotide containing the

(13) Dähne, S.; Freyer, W.; Teuchner, K.; Dobkowski, J.; Grabowski, Z. R. *J. Lumin.* **1980**, *22*, 37–49.

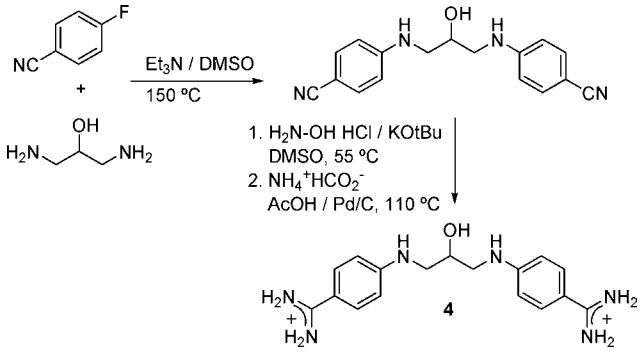
(14) (a) Nguyen, B.; Neidle, S.; Wilson, W. D. *Acc. Chem. Res.* **2009**, *42*, 11–21. (b) Degtyareva, N. N.; Wallace, B. D.; Bryant, A. R.; Loo, K. M.; Petti, J. T. *Biophys. J.* **2007**, *92*, 959–965.

(15) For data treatment, details, and derivation of the mathematical expressions, see the Supporting Information.

(16) Chaires, J. B. *Arch. Biochem. Biophys.* **2006**, *453*, 26–31.

(17) Munde, M.; Ismail, M. A.; Arafa, R.; Peixoto, P.; Collar, C. J.; Liu, Y.; Hu, L.; David-Cordonnier, M.-H.; Lansiaux, A.; Bailly, C.; Boykin, D. W.; Wilson, W. D. *J. Am. Chem. Soc.* **2007**, *129*, 13732–13743.

Scheme 2. Synthesis of Nucleophilic BAPPA **4**



selected DNA sequence was titrated with increased amounts of the nonfluorescent minor groove binder. The fluorescence emission spectra were recorded after each addition, and the reduction in fluorescence resulting from the increased displacement of the fluorescent BAPPA from the minor groove was used to obtain the binding constant of the DNA binder.[18]

We routinely used concentrations of 0.5 μM BAPPA and 1.4 μM dsDNA in these experiments and the concentration of the external binder up to 150−500 μM, depending on its relative binding affinity for the DNA. While propamidine itself shows an affinity for the *AATTT* site similar to that of BAPPA, introduction of an amino group in the middle of the alkyl chain linker (analogue **5**, Figure 4) led to a slight decrease in DNA binding affinity. Interestingly, replacing the amidinium group in BAPPA with a guanidinium group also induced a near 10-fold decrease in affinity (analogue **6**, Figure 4).

We have also carried out preliminary tests of the in vivo internalization and fluorescence emission properties of the conjugation friendly analogue **4**, using HeLa cells. We were able to observe reasonable bright fluorescence emission concentrated in the cell nuclei and clearly differentiated from the autofluorescence background (see the Supporting Information for details).

In summary, *aza*-propamidine BAPPA is a structurally simple and efficient fluorescent probe for A/T-rich dsDNA

(18) For competitive displacement analysis, see: (a) Roehrl, M. H. A.; Wang, J. Y.; Wagner, G. *Biochemistry* **2004**, *43*, 16056–16066. (b) Wang, Z.-X. *FEBS Lett.* **1995**, *360*, 111–114.

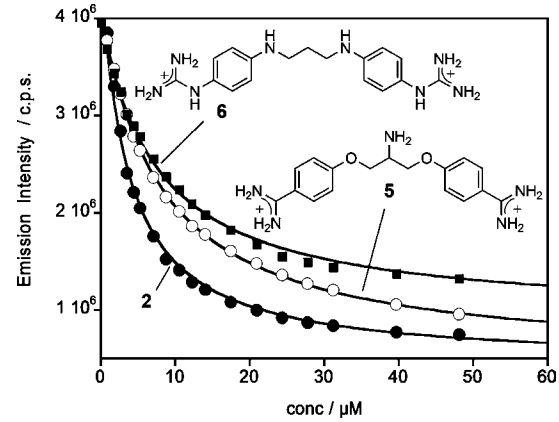


Figure 4. Displacement curves of 0.5 μM BAPPA and 1.4 μM of hairpin oligonucleotide containing the *AATTT* site with: propamidine **2** (●, $K_D = 161 \pm 22$ nM), aminopropamidine **5** (○, $K_D = 452 \pm 7$ nM), and guanidine analogue **6** (■, $K_D = 479 \pm 7$ nM). Emission monitored at 387 nm.

sites, displaying a remarkable fluorescence emission enhancement upon binding to the minor groove of such sequences. This intrinsic DNA-dependent fluorogenicity, together with the good cell translocation properties, suggests that BAPPA and its derivatives might offer great potential for the future development of dsDNA-dependent chemical, optical, and biological applications.

Acknowledgment. This work is dedicated to Prof. Luis Castedo on the occasion of his 70th birthday. We thank the support given by the Spanish grants SAF2007-61015, SAF2006-06868, CTQ2006-01339, *Consolider Ingenio* 2010 CSD2007-00006, and the Xunta de Galicia PGIDIT06PXIB209018PR, GRC2006/132, and PGIDIT08CSA047209PR. O.V. and M.I.S. thank the Spanish MICINN for their PhD fellowships. M.E.V. also thanks the *Human Frontier Science Program* (CDA0032/2005-C) and the Spanish MICINN for his *Ramon y Cajal* contract.

Supporting Information Available: Synthesis, characterization, DNA binding, cell uptake experiments, and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL902501J