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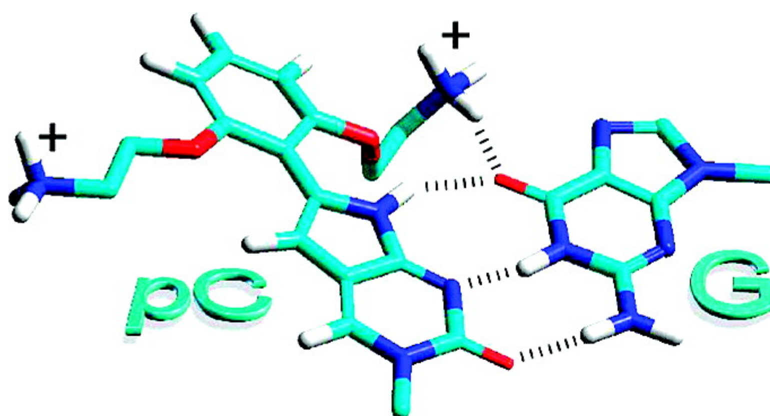

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Fluorescence and Hybridization Properties of Peptide Nucleic Acid Containing a Substituted Phenylpyrrolocytosine Designed to Engage Guanine with an Additional H-Bond

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Over the past decade fluorescent DNA base analogues have received considerable attention, and there continues to be a growing interest in their design, study, and application.[1] Fluorescent nucleobase analogues that maintain fidelity of base-pairing, possess a high fluorescence quantum yield, and undergo a significant change in quantum yield upon hybridization are prized for use in the study of nucleic acid conformation/dynamics and as hybridization probes.

The archetypal responsive fluorescent nucleobase is the adenine analogue 2-aminopurine which retains complementarity to thymine/uracil but also wobble pairs with cytosine. It has an exceptionally high fluorescence quantum yield ($\Phi_f = 0.68$) that decreases when incorporated into oligonucleotides owing to base-stacking interactions[2] and thus has found use as a probe of environmental changes. There exists relatively few such fluorescent nucleobases complementary to other nucleosides; fewer yet have been incorporated into oligonucleotides or analogues.[3] With respect to the recognition of guanine, the most studied examples are the tricyclic phenothiazine analogue of cytosine (tC),[4] its oxygen-containing homologue phenoxazine (tC°),[5] and 6-methylpyrrolocytosine (MepC).[6] The tC base has been studied in both DNA and peptide nucleic acid (PNA) and its high fluorescence ($\Phi_f = 0.20$) has been reported to be insensitive to the single or double stranded nature of the DNA and the base sequence.[7] The tC° analogue, when incorporated into DNA, has an average quantum yield of 0.22 and has been referred to as the brightest DNA-incorporated base analogue reported so far.[5] Although pyrrolocytosine derivatives have been incorporated into DNA and RNA and demonstrate responsiveness to hybridization, these fluorophores are relatively inefficient ($\Phi_f \approx 0.06$).[8]

Oligonucleotide analogues capable of forming highly stable complexes with their target nucleic acids can be productively combined with fluorescent labels to construct specific, high affinity probes as potential bioanalytical reagents.[9] One such oligonucleotide analogue is peptide nucleic acid (PNA).[10] The increased affinity is useful for stabilizing relatively short probes or to out compete other ligands for the same target sequence. Herein, we describe an exceptionally bright and environmentally sensitive pyrrolocytosine nucleobase analogue designed for tight binding to guanine and its hybridization performance in PNA.

We have previously described the synthesis and fluorescence emission properties of some para-substituted 6-phenylpyrrolocytosine (PhpC) derivatives[11] and report herein a new cytosine base analogue, [bis-*ortho*-(aminoethoxy)phenyl]pyrrolocytosine (boPhpC), that is designed to engage guanine with an additional hydrogen bond, Figure 1.[12] The hybridization properties, fluorescence quantum yields, and fluorescence response to cDNA and RNA are reported.

A domino Sonogashira cross-coupling/annulation reaction sequence with an appropriately derivatized terminal alkyne was employed as the key step in the synthesis of the bicyclic nucleobase luminophore (see Supporting Information). The dual appendages

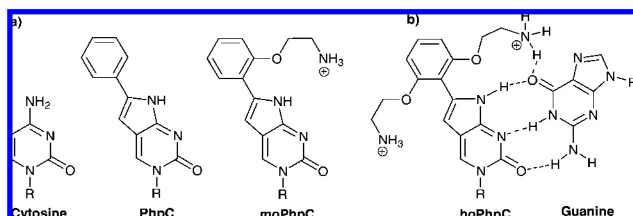

Figure 1. (a) Structures of nucleobases studied; cytosine, 6-phenylpyrrolocytosine (PhpC), [mono-*o*-(aminoethoxy)phenyl]pyrrolocytosine (moPhpC), and [bis-*o*-(aminoethoxy)phenyl]pyrrolocytosine (boPhpC). (b) Proposed interaction of boPhpC with guanine.

Table 1. T_m Data (°C) for PNA:DNA and PNA:RNA Duplexes, Duplex Stabilization (ΔT_m) and Mismatch Discrimination for DNA[a]

		match		mismatch (DNA target)		
sequence	nucleobase	DNA	RNA	Z = C	Z = A	Z = T
1	X = cytosine	49.5	57.0	(−7.5)	(−4.5)	(−13.5)
2	X = PhpC	(+2.5)	(−2.0)	(−13.0)	(−7.0)	(−9.0)
3	X = moPhpC	(+10.5)	(+4.0)	*b*	*b*	*b*
4	X = boPhpC	(+9.5)	(+3.0)	(−15.0)	(−16.0)	(−16.0)
5	Y = cytosine	51.0	63.0	(−15.5)	(−14.0)	(−15.0)
6	Y = PhpC	(+2.0)	(+1.0)	(−11.0)	*b*	*b*
7	Y = moPhpC	(+7.0)	(+3.0)	(−11.0)	(−9.0)	(−11.0)
8	Y = boPhpC	(+11.5)	(+10.0)	(−13.5)	*b*	(−14.5)

[a] PNA sequences: GTA GAT **X**AC T-Lys and GTA GAT C**Y**C T-Lys. Z = mismatch base in DNA. [b] No observed cooperative transition. ΔT_m values in parentheses, positive values indicate duplex stabilization.

on the phenyl ring serve the purpose of increasing the aqueous solubility of the modified insert and providing a Hoogsteen hydrogen bond to O(6) of guanine.[13,14] For comparison, unsubstituted (PhpC) and monosubstituted (moPhpC) pyrrolocytosine-containing PNAs were also prepared, Figure 1.

The hybridization properties of sequences **1**−**8**[12b] were evaluated with DNA and RNA and compared with the control oligomers (X and Y = cytosine), Table 1. Incorporation of PhpC into oligomers resulted in a consistently modest increase in T_m (ca. +2.0 °C with DNA) ascribed to increased π-stacking. Incorporation of boPhpC resulted in a marked increase in binding affinity (+9.5 and +11.5 °C, with DNA) presumably due, in large part, to an additional hydrogen bond to guanine. Only **8**, containing boPhpC, exhibited a significant increase in affinity toward RNA. Such dramatic sequence effects are also observed for other related cytosine nucleobase analogues.[12]

The formation of helical complexes was confirmed by circular dichroism spectrophotometry. While single-stranded PNA (**1**−**8**) characteristically showed no CD signal; complexes with cDNA for all fully matched heteroduplexes (PNA:DNA) displayed a positive Cotton effect at 260 nm and a shoulder at 280 nm and another positive peak at ca. 220 nm. The similarity of spectral features for heteroduplexes formed whether possessing an unmodified cytosine, PhpC,

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Table 2. Photophysical Data of boPhpC[a]

	Exc, λ_{max} (nm)	Em, λ_{max} (nm)	Φ_f[b]
dioxane	378	455	0.75
1-octanol	379	461	0.74
acetone	378	456	0.62
ethanol	372	461	0.61
aqueous buffer[c]	365	468	0.32

[a] Referring to Figure 1, fluorophore structure R = CH_2CO_2Et. [b] Quantum yield determination detailed in ESI. [c] Consists of 100 mM NaCl, 10 mM Na_2PO_4, 0.1 mM EDTA, pH 7.

moPhpC, or boPhpC suggests similar global conformations. Importantly, the CD spectra clearly showed the formation of duplexes possessing a mismatched basepair with an approximately 15 nm bathochromic shift of the 260 nm band. All PNA also formed helical complexes with complementary RNA (see Supporting Information).

To establish whether the increased affinity toward cDNA or RNA was due to the additional hydrogen bond to guanine or electrostatic interactions, moPhpC was synthesized. If electrostatic interactions were the major source of increased duplex stability, moPhpC possessing only one amino group should hybridize with lower affinity than boPhpC. However, this is not the case, as moPhpC yields a hybrid with similar thermal stability (cf. **3** and **4**) suggesting that the increased affinity is not solely due to electrostatic interactions but due to specific and discriminating bonding interactions to guanine.

The excellent base discrimination ability of boPhpC is illustrated by using singly mismatched DNA (Table 1). In both PNA sequences a mismatch was detrimental to duplex stability as indicated by the decrease in T_m ($\Delta T_m \geq -13.5$ °C). This discrimination is equivalent to or better than cytosine itself.

Next, we investigated the fluorescence properties of boPhpC because of its demonstrated excellent binding properties and increased PNA solubility. The photophysical study of boPhpC revealed an exceptional quantum yield which was sensitive to the molecular environment. Although the quantum yield is strongly influenced by the environment and decreases with increasing the polarity of the medium (Table 2), it remains remarkably high in aqueous solution. The value of $\Phi_f = 0.32$ ($\epsilon_{365} = 6650$) for the boPhpC luminophore is comparable to the best fluorescent cytosine analogues yet reported.

The effect of the medium on the fluorescence was also examined by studying water/ethanol mixtures. As the polarity of the medium decreases, there is a smooth increase in the fluorescence emission which correlates to the changes measured for the quantum yields in different solvents (see Supporting Information).

Having established that boPhpC was bright,[15] responsive to the medium, and a discriminating base-pairing partner, its fluorescence properties in the single- and double-stranded state were compared. It is notable that the quantum yield of the PNA single strand is greater than the free fluorophore indicating that the boPhpC nucleobase is in a molecular environment that more resembles a medium of low polarity. Upon duplex formation, in both sequence contexts, the quantum yield of the boPhpC contained within the PNA strand dramatically decreases by ca. 50% or greater than a 2-fold change of maximal emission intensity, Figure 2. The quenching of fluorescence upon duplex formation is consonant with the behavior of MepC and PhpC in DNA and RNA and likely has both base stacking interactions and H-bonding components.[6,16]

In summary, we have prepared a novel fluorescent nucleobase that possesses an exceptionally high quantum yield. The fluorophore is environmentally sensitive and is able to report on PNA/DNA hybridization. The modified base also demonstrated high affinity binding to guanine with excellent base recognition specificity. We anticipate that this modification will find use as a valuable reporter group in PNA-based hybridization probes and in potential antisense/antigene applications.

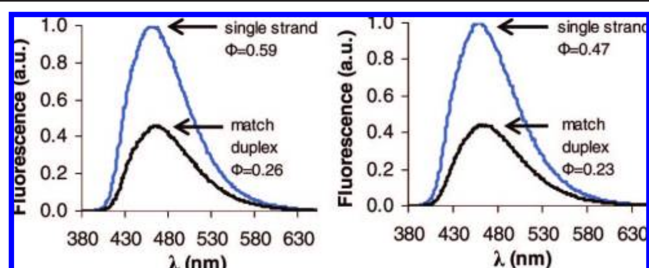

Figure 2. Fluorescence emission spectra and quantum yield of sequence **4** (left) and sequence **8** (right), each containing the boPhpC modification.

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Supporting Information Available: Characterization data, synthetic procedures, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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