ORGANIC LETTERS

2009
Vol. 11, No. 21
4878−4881

Peptide Nucleic Acid Containing a Meta-Substituted Phenylpyrrolocytosine Exhibits a Fluorescence Response and Increased Binding Affinity toward RNA

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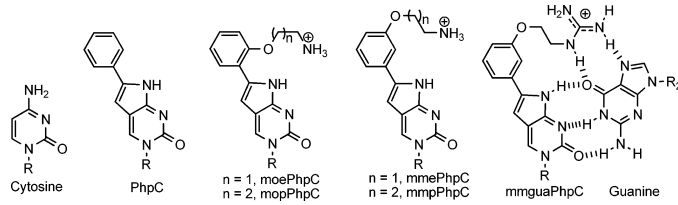
Received August 21, 2009

ABSTRACT



Peptide nucleic acids (PNA) containing meta-substituted 6-phenylpyrrolocytosine (PhpC), [mono-*m*-(aminoethoxy)phenyl]pyrrolocytosine (mmePhpC), [mono-*m*-(aminopropoxy)phenyl]pyrrolocytosine (mmpPhpC), and [mono-*m*-(guanidinoethoxy)phenyl]pyrrolocytosine (mmguaPhpC), have been synthesized. Meta-substituted PhpCs have been hybridized with overall higher binding affinity toward DNA and RNA than previously synthesized moePhpC or newly synthesized mopPhpC. The guanidinium-containing nucleobase, mmguaPhpC, exhibited the highest increase in binding affinity toward RNA while fluorometrically responding on the state of hybridization.

The design and study of fluorescent nucleobase analogues continues to be of interest as they find use in numerous biotechnological and biomedical applications.[1] Many of these applications would also benefit from increased binding affinity between the oligonucleotide anologue and the target nucleic acid.[2] The nucleic acid complex is governed by Watson−Crick hydrogen bonding as a source of complementary recognition and is further stabilized by $\pi-\pi$ stacking. Introducing modified nucleobases with improved stacking interactions and hydrogen bonding is thereby a viable approach to increasing binding affinity in a sequence-discriminating manner. For some applications, it is desirable not only to increase the binding affinity but also to construct a nucleobase that concurrently undergoes a fluorescent response upon hybridization or change in environment.

Peptide nucleic acid (PNA) is an oligonucleotide analogue capable of forming highly stable complexes with its target nucleic acid.[3] Numerous modified nucleobases have been incorporated into PNA.[4] Two notable fluorescent cytosine analogues are phenoxazine[5] and phenylpyrrolocytosine (PhpC).[6] When containing an aminoethoxy moiety, the phenoxazine nucleobase forms a fourth hydrogen bond to guanine and has been termed the G-clamp[7] (Figure 1a). Both the phenoxazine

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10.1021/ol9019474 CCC: $40.75
Published on Web 09/29/2009

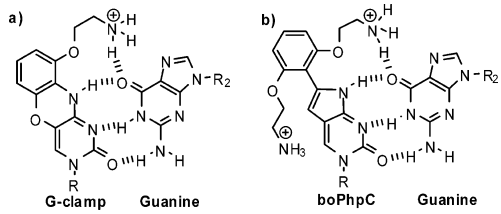


Figure 1. Previously studied cytosine analogues, based on the phenoxazine and PhpC nucleobases, capable of forming a fourth hydrogen bond to guanine: (a) phenoxazine-based G-clamp and (b) phenylpyrrolocytosine-based boPhpC.

and G-clamp nucleobases continue to be modified and exploited in various applications.[8]

We have recently designed a novel intrinsically fluorescent cytosine analogue based on PhpC, termed boPhpC[9] (Figure 1b).

Our previous work indicated that the boPhpC nucleobase also formed a fourth hydrogen bond to guanine, thereby increasing binding affinity, but also fluorimetrically reported on PNA/DNA hybridization.[9] This dual behavior places boPhpC and its analogues in a unique class of molecules.

While studying the hybridization properties of moePhpC (Figure 2a), we noticed significantly greater stabilization of

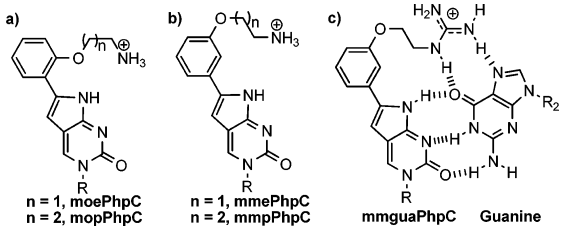


Figure 2. Structures of modified PhpCs designed to engage guanine with an additional hydrogen bond. (a) Previously studied moePhpC and newly synthesized mopPhpC; (b) meta-substituted PhpCs mmePhpC ($n = 1$) and mmpPhpC ($n = 2$); (c) proposed interaction of guanidino-PhpC (mmguaPhpC) with guanine.

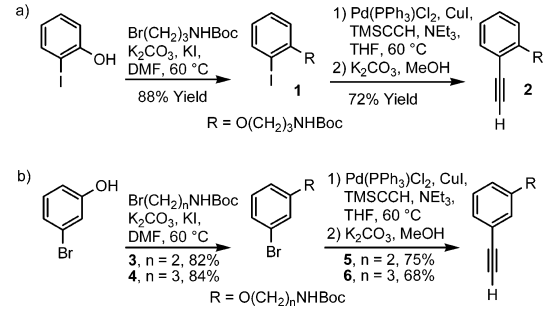
a PNA/DNA duplex over a PNA/RNA duplex of the same sequence. Furthermore, the flanking nucleobases in PNAs containing moePhpC would affect the overall duplex stability. In a recent study, an unusual sequence-dependent behavior has also been observed with the G-clamp in both DNA and PNA.[10]

In order to address the lower binding affinity toward RNA and the sequence-dependent behavior, we have decided to investigate second generation PhpCs by varying the position and nature of substitution on the phenyl ring. It was our intention in the design that the bases remain capable of forming an additional hydrogen bond to guanine while fluorometrically responding on the state of hybridization (Figure 2).

We first decided to synthesize the ortho-substituted analogue with a three-carbon chain, mopPhpC (Figure 2a), in order to establish if the ethyl linker (moePhpC) was too short. Further, we reasoned that ortho-substituted PhpCs may form an undesirable secondary repulsive interaction between the oxygen on the phenyl ring and the O6 of guanine. The meta-substituted PhpCs (mmePhpC and mmpPhpC, Figure 2b) were synthesized in order to determine if this substitution was more favorable, potentially by relief of secondary repulsive interactions. Finally, we chose the nucleobase with the highest affinity toward both DNA and RNA as a good candidate for derivatiztion to the guanidinium group. Installation of the guanidinium group on the mmePhpC scaffold holds the potenial to further increase the binding affinity through dual O6- and N7-Hoogsteen hydrogen bonds (Figure 2c).

The required alkyne, **2**, for the synthesis of mopPhpC was prepared by treating 2-iodophenol with *tert*-butyl 2-bromopropylcarbamate to give **1** which was subjected to Sonogashira reaction conditions and TMS removal to give the terminal alkyne **2** (Scheme 1a). Similarly, the synthesis

Scheme 1. Synthesis of Alkynes **2**, **5**, and **6**



of alkynes **5** and **6** was accomplished by treating 3-bromophenol with *tert*-butyl 2-bromoethylcarbamate or *tert*-butyl 2-bromopropylcarbamate to give **3** or**4**, which were subjected to Sonogashira reaction conditions and TMS removal to give alkynes **5** and **6** (Scheme 1b).

Alkynes **2**, **5**, or **6** and ethyl (N^4-benzoyl-5-iodocytosin-1-yl)acetate[6a] were subjected to a one-pot Sonogashira cross-

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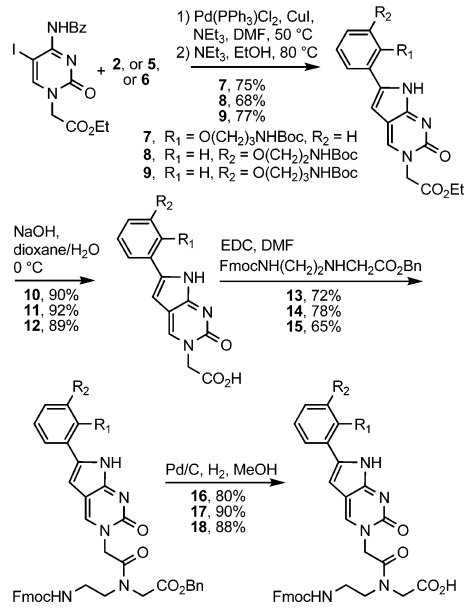
coupling/annulation reaction to give **7**, **8**, or **9** in good yield (Scheme 2).

Scheme 2. Synthesis of Substituted PhpCs **16**−**18**



Hydrolysis of the ester group followed by carbodiimide-mediated condensation with *N*-[2-(Fmoc)aminoethyl]glycine benzyl ester[11] gave the monomer ester **13**, **14**, or **15**. The benzyl ester was hydrogenated to give the final monomers **16** (mopPhpC), **17** (mmePhpC), or **18** (mmpPhpC).

An orthogonally protected PNA monomer containing a protected guanadinium group (**22**) was synthesized by treating **8** with TFA, followed by guanylation of the intermediate amine with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine in the presence of DIPEA. The monomer, mmguaPhpC **22**, was synthesized similarly to the above synthesized monomers (Scheme 3).

Monomers **16**, **17**, **18**, and **22** were incorporated into two PNA oligomers; GTA GAT **X**AC T-Lys and GTA GAT C**Y**C T-Lys. The oligomers were cleaved from the solid support by standard conditions, purified by RP-HPLC and characterized by ESI-MS (see the Supporting Information).

The hybridization properties of previously synthesized sequences (**1**−**3** and **8**) containing cytosine,[9a] PhpC,[9a] and moePhpC[9a] were compared to newly synthesized sequences (**4**−**7** and **11**−**14**) containing mopPhpC, mmePhpC, mmpPhpC, and mmguaPhpC (Table 1). A decrease in binding affinity was observed when mopPhpC was incorporated into the sequence **4** (+2.5 °C with DNA and +1.0 °C with RNA, seq **4**) compared to moePhpC (+10.5 °C with DNA and +4.0 °C with RNA, seq **3**). However the propyl linker performed better in seq **11**, which resulted in an increase in binding affinity (+11.0 °C with DNA and +7.0 °C with RNA, seq **11**) compared to moePhpC (+7.0 °C with DNA and +3.0 °C with RNA, seq **10**). Therefore increasing the tether length in ortho-substituted PhpC does not eliminate sequence dependent binding affinity nor does it increase the overall binding affinity toward RNA.

Scheme 3. Synthesis of mmguaPhpC Monomer

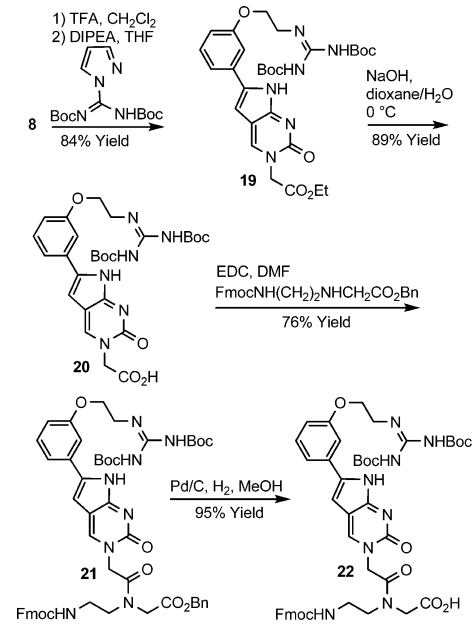


Table 1. T_m Data (°C) for PNA:DNA and PNA:RNA Duplexes[a]

		DNA		RNA	
seq	nucleobase	T_m	ΔT_m	T_m	ΔT_m
1[9a]	**X** = cytosine	49.5		57.0	
2[9a]	**X** = PhpC	52.0	+2.5	55.0	−2.0
3[9a]	**X** = moePhpC	60.0	+10.5	61.0	+4.0
4	**X** = mopPhpC	52.0	+2.5	58.0	+1.0
5	**X** = mmePhpC	63.0	+13.5	63.0	+6.0
6	**X** = mmpPhpC	60.0	+10.5	61.0	+4.0
7	**X** = mmguaPhpC	60.0	+10.5	63.5	+6.5
8[9a]	**Y** = cytosine	51.0		51.0	
9[9a]	**Y** = PhpC	53.0	+2.0	52.0	+1.0
10[9a]	**Y** = moePhpC	58.0	+7.0	54.0	+3.0
11	**Y** = mopPhpC	62.0	+11.0	58.0	+7.0
12	**Y** = mmePhpC	61.5	+10.5	59.0	+8.0
13	**Y** = mmpPhpC	64.5	+13.5	54.0	+3.0
14	**Y** = mmguaPhpC	63.0	+12.0	63.0	+12.0

[a] Sequences: GTA GAT **X**AC T-Lys and GTA GAT C**Y**C T-Lys.

The introduction of meta-substituted PhpCs, mmePhpC and mmpPhpC revealed very promising results. In both oligomers an increase in binding affinity toward RNA was observed. Likewise, a $\Delta T_m > 10.0$ °C was maintained for DNA in both sequences.

An average melting temperature, $\Delta T_{m(avg)}$, for the modified nucleobases is shown in Table 2. This represents a measure of the consistency of performance of the base modifica-

(11) Wojciechowski, F.; Hudson, R. H. E. *J. Org. Chem.* **2008**, *73*, 3807–3816.

Table 2. Average Melting Temperature $\Delta T_{m(avg)}$ (°C) for PNA:DNA and PNA:RNA Containing the Modified Nucleobases[a]

nucleobase	DNA ($\Delta T_{m(avg)}$)	RNA ($\Delta T_{m(avg)}$)
cytosine		
PhpC	+2.25	−0.5
moePhpC	+8.8	+3.5
mopPhpC	+6.75	+4.0
mmePhpC	+12.0	+7.0
mmpPhpC	+12.0	+5.5
mmguaPhpC	+11.25	+9.25

[a] Average ΔT_m values are the sum of the ΔT_m values from both sequences in Table 1 divided by two.

tion in different sequence contexts. The meta-substituted mmePhpC hybridized with a $\Delta T_{m(avg)}$ of +12.0 with DNA and +7.0 with RNA. Likewise the propyl tether in mmpPhpC hybridized with high affinity ($\Delta T_{m(avg)}$ +12.0 °C with DNA) but slightly lower toward RNA ($\Delta T_{m(avg)}$ +5.5 °C). Therefore, mmePhpC was chosen for derivatization to the guanidinium-containing nucleobase.

The introduction of mmeguaPhpC into sequence **7** and **14** resulted in high affinity binding toward DNA (+10.5 °C and +12.0 °C, $\Delta T_{m(avg)}$ +11.25) but also exhibited a significant increase in binding affinity toward RNA (+6.5 °C and +12.0 °C, $\Delta T_{m(avg)}$ +9.25).

The fluorescence responses of sequences **4**−**7** and **11**−**14** were evaluated against DNA and RNA (Supporting Information). The fluorescence response of sequences containing mmguaPhpC in the single stranded state and hybridized to DNA or RNA is shown in Figure 3. A 60% decrease in emission intensity is observed in sequence **7**,but only a 35% decrease in intensity is observed in sequence **14** when hybridized to DNA.

The fluorescence response of oligomers containing mmguaPhpC is better with RNA; upon duplex formation, a 50% decrease in emission intensity is observed in sequence **7** and a 70% in sequence **14** (Figure 3). The quenching of fluorescence upon duplex formation is consistent with MepC and PhpC in DNA and RNA and is likely due to both hydrogen-bonding and base-stacking interactions.[6b,12]

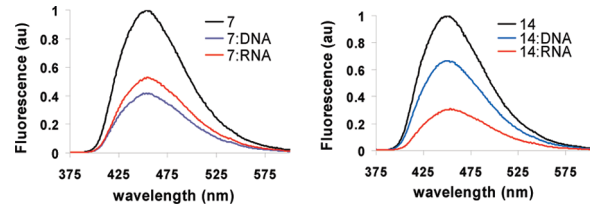


Figure 3. Fluorescence emission spectra of sequences **7** and **14** containing mmguaPhpC when hybridized to DNA and RNA.

In summary, we have shown that rational modification of PhpC produces high-affinity cytosine analogues potentially capable of forming a fourth and fifth hydrogen bond to guanine while fluorometrically reporting on the state of hybridization. We have also shown that switching the aminoethoxy or aminopropoxy tether from the ortho position to the meta position in PhpC maintains high affinity binding to DNA but, more importantly, significantly increases the binding affinity toward RNA. Preliminary data also show that meta-substituted PhpC may alleviate unusual sequence-dependent binding affinity such as that shown with the G-clamp.

Future work will be aimed at evaluating the binding affinty of oligonucleotide analogues containing meta-substituted PhpC in various sequence contexts. These nucleobases will also be evaluated in antisense or antigomer applications or as fluorescent-based hybridization probes.

Acknowledgment. We thank the Natural Science and Engineering Research Council of Canada for support of this work including a PGS-D scholarship (F.W.).

Supporting Information Available: Characterization data and experimental procedures for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL9019474

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