ORGANIC LETTERS

2002
Vol. 4, No. 25
4395−4398

High-Affinity Peptide Nucleic Acid Oligomers Containing Tricyclic Cytosine Analogues†

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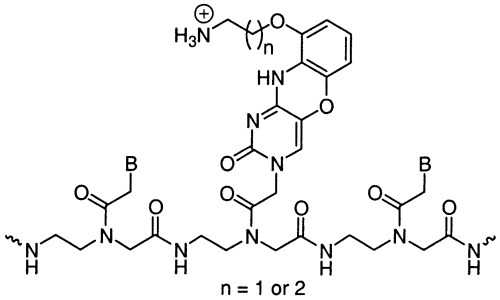
Received August 6, 2002 *(Revised Manuscript Received October 31, 2002)*

ABSTRACT



Peptide nucleic acid (PNA) monomers containing the tricyclic cytosine analogues phenoxazine, 9-(2-aminoethoxy)phenoxazine (G-clamp), and 9-(3-aminopropoxy)phenoxazine (propyl-G-clamp) have been synthesized. The modified nucleobases were incorporated into PNA oligomers using Boc-chemistry for solid-phase synthesis. PNAs containing single G-clamp modifications exhibit significantly enhanced affinity toward RNA and DNA targets relative to unmodified PNA while maintaining mismatch discrimination. These PNA G-clamp modifications exhibit the highest increase in affinity toward nucleic acid targets reported so far for PNA modifications.

In peptide nucleic acids (PNAs) the sugar phosphate backbone of an oligonucleotide is replaced by a pseudopeptide backbone derived from *N*-(2-aminoethyl)glycine.[1,2] Unfortunately, certain PNA sequences have poor solubility and unmodified PNAs have limited cellular uptake and a tendency for self-aggregation.[3] These problems have been addressed by introducing PNA backbones carrying charges,[2,4,5] by preparing chimeras of PNA with nucleic acids,[6] peptides,[7] and polyamines,[8] and by introducing hydrophilic amino acids[9] into the backbone of PNA.

In addition to the natural nucleobases, a number of heterocyclic modifications on the PNA backbone have been reported in the literature including 2,6-diaminopurine,[10,11] pseudoisocytosine,[12] 5-bromouracil,[13,14] 5-iodouracil,[15] 4-thio-

† Presented in part at the 222nd National Meeting of the American Chemical Society, August 26−30, 2001 Chicago, IL, 2001; American Chemical Society: Washington, DC, 2001; Abstracts of Papers (CARB-015). Rajeev, K. G.; Maier, M. A.; Manoharan, M. Modified nucleobase containing peptide nucleic acids (PNAs). US patent application filed May 25, 2001, Isis Pharmaceuticals, Inc.

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10.1021/ol027026a CCC: $22.00
Published on Web 11/15/2002

thymine,[16] 2-thiouracil,[17,18] 5-methyl-4-(*3H*-(1,2,4)triazol-1-yl)-3,4-dihydro-*1H*-pyrimidine-2-one,[19] and 5-methylcytosine.[12] Incorporation of a tricyclic 3,5-diaza-4-oxophenothiazine into PNA showed an affinity increase of 3 °C per modification with complementary DNA or RNA as compared to unmodified PNA.[20] PNAs containing 1,8-naphthyridin-2(1*H*)-one and benzo[*b*]-1,8-naphthyridin-2(1*H*)-one as substitutes for thymine have also been reported.[20,21] For each of these modifications, the increase in thermal binding was rather small to modest. We became interested in the design of heterocyclic modifications that could enhance the binding affinity to mRNA, increase solubility, and increase the cellular uptake of PNA.

Several tricyclic cytosine analogues have been synthesized. These include 9-(2-aminoethoxy)phenoxazine, called the G-clamp (1, Figure 1), that carries an aminoethoxy moiety

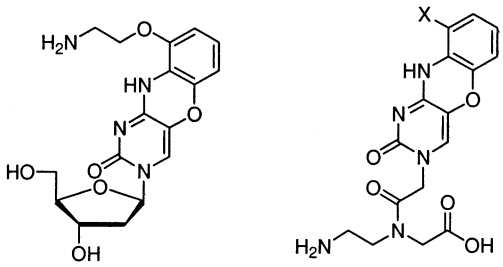

Figure 1. Structure of monomeric DNA and PNA G-clamp.

attached to the rigid phenoxazine scaffold.[22,23] Incorporated into oligonucleotides, these cytosine modifications hybridize with guanine and enhance duplex stability through extended stacking interactions. Binding studies demonstrated that a single incorporation of the G-clamp enhanced the binding affinity of an oligodeoxynucleotide (ODN) to its complementary target DNA or RNA with a ΔT_m of up to 18 °C relative to 5-methylcytosine (dC5Me). It was suggested that the tethered amino group formed an additional hydrogen bond with O6 in the Hoogsteen face of a complementary guanine.[22] It has been shown by X-ray crystal structural analysis of a oligonucleotide decamer duplex containing a guanidino G-clamp, an analogue of G-clamp, that a Hoogsteen-type hydrogen bond does exist between O6 and N7 of G and the tethered guanidino group.[24] This suggests that the increased affinity of G-clamp is mediated by the combination of extended base stacking and at least one additional specific hydrogen bond between O6 of G and the tethered amino group. Single incorporation of the G-clamp at the 3′-terminus of an ODN resulted in a significant increase in nuclease stability as well.[25] Biological studies on G-clamp-containing phosphorothioate ODNs for specific antisense targets such as cyclin-dependent kinase inhibitor, $p27^{kip1}$, and *c*-raf showed enhanced potency relative to unmodified ODN.[23]

Given the benefits the G-clamp modification offers for antisense phosphorothioate oligonucleotides, we envisioned that introduction of the G-clamp and its derivatives into PNA would improve the molecular recognition properties of PNA. We reasoned that the additional positive charge on the modified base might have a positive impact on solubility and cellular uptake properties of the modified PNAs.

In the present Letter we describe convenient syntheses of suitably protected tricyclic phenoxazine (**2a**), G-clamp (**2b**), and 9-(3-aminopropoxy)phenoxazine (propyl G-clamp, **2c**) PNA monomers shown in Figure 1. Each monomer has been incorporated into PNA using Boc-chemistry and solid-phase synthesis and deprotection protocols. Also reported are preliminary accounts of the hybridization behavior of these analogues with complementary DNA and RNA, and mismatched RNA strands.

An orthogonally protected G-clamp PNA monomer (**10a**) was synthesized from 5-bromouracil (**3**) as shown in Scheme 1. Trimethylsilylation of 5-bromouracil in HMDS under reflux followed by treatment of the silylated derivative with ethyl bromoacetate in refluxing acetonitrile yielded 5-bromo-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidine-acetic acid ethyl ester (**4**) in quantitative yield. Activation at C4 of compound **4** by treatment with $POCl_3$ and triazole in the presence of triethylamine and subsequent substitution of the triazole moiety at C4 with 2-aminoresorcinol in the presence of diisopropylethylamine yielded compound **5** in 76% yield. Compound **5** was then subjected to monoalkylation with benzyl *N*-(2-hydroxyethyl)carbamate under Mitsunobu alkylation conditions in acetonitrile to obtain compound **6a** in 80% yield.[22] Treatment of compound **6a** with a 10 molar excess of cesium fluoride[26] and one molar equiv of Cs_2CO_3 in absolute ethanol under reflux for 36 h gave a mixture of ethyl ester **7a** and its corresponding carboxylic acid **8a**. Compound **8a** was precipitated from the aqueous extract of the reaction by the addition of $KHSO_4$. The ethyl ester **7a** was stirred with LiOH at a temperature below 10 °C and

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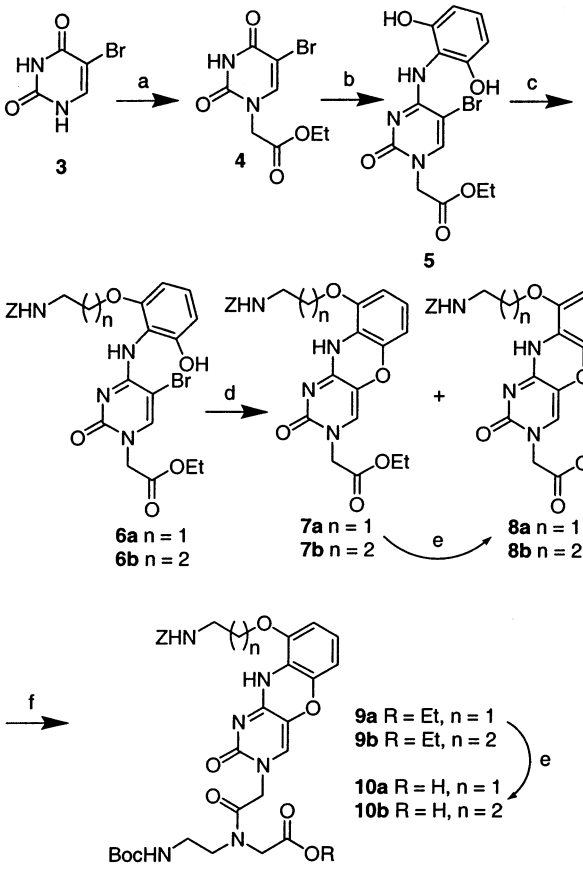
Scheme 1[a]



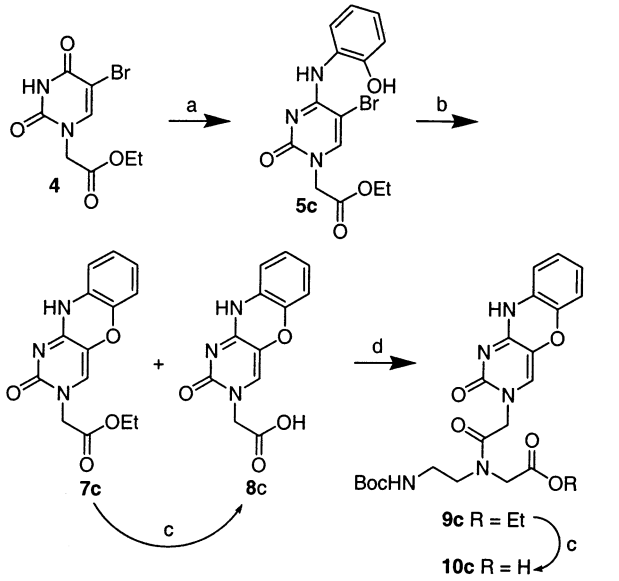
[a] Reagents and conditions: (a) (i) HMDS, reflux, (ii) ethylbromoacetate, MeCN reflux (94%); (b). (i) $POCl_3$, triazole, TEA/MeCN, −10 °C, (ii) 2-aminoresorcinol, DIEA/DCM, rt, 6 h (76%); (c) Ph_3P, DEAD, $ZHN(CH_2)_nOH$ (n = 2 or 3)/MeCN, 24 h (80%); (d) CsF (10 equiv), Cs_2CO_3 (1 equiv)/EtOH, reflux 36 h (83%); (e) (i) LiOH, (ii) $KHSO_4$ (>95%); (f) $(Boc)HNCH_2CH_2NH(CH_2COOEt)$, DCC, DhbhOH, DMAP/DMF, 12 h (50%).

subsequent addition of $KHSO_4$ gave the free carboxylic acid **8a** in quantitative yield.

The carboxylic acid **8a** was coupled to ethyl *N*-(2-*tert*-butyloxycarbonylaminoethyl)glycinate via prior activation of the carboxylic group with DCC and DhbhOH to obtain the fully protected G-clamp monomer **9a** in 52% yield.[11] Saponification of **9a** at low temperature using LiOH yielded the desired PNA monomer **10a**. The propyl G-clamp monomer (**10b)** was obtained from compound **5** via monoalkylation with benzyl *N*-(3-hydroxypropyl)carbamate under Mitsunobu alkylation conditions in acetonitrile followed by subsequent reactions as described for the synthesis of compound **10a**.

The phenoxazine PNA monomer (**10c**) was synthesized from compound **4** as shown in Scheme 2. Unlike the ring closure reaction in the tricyclic systems that have the aminoalkyl tethers (**10a** and **10b**), the ring closure of compound **5c** with cesium fluoride and cesium carbonate to obtain compound **7c** or **8c** was very sluggish. For this reason, without purifying the ester **7c**, it was saponified to **8c** and subsequently coupled to ethyl *N*-(2-*tert*-butyloxycarbonyl-

Scheme 2[a]



[a] Reagents and conditions: (a) (i) $POCl_3$, triazole, TEA/MeCN, −10 °C, (ii) 2-aminophenol, DIEA/DCM, rt, 6 h (51.7%); (b) CsF (10 equiv), Cs_2CO_3 (1 equiv)/EtOH, reflux 36 h; (c) (i) LiOH, (ii) $KHSO_4$; (d) $(Boc)HNCH_2CH_2NH(CH_2COOEt)$, DCC, DhbhOH, DMAP/DMF, 12 h (6%, over all yield from **5c**).

aminoethyl)glycinate as described for the synthesis of compound **9a** to obtain the fully protected monomer **9c** in 6% over all yield. Alkaline hydrolysis of compound **9c** gave the desired phenoxazine PNA monomer **10c** in quantitative yield.

PNA synthesis was performed using Boc chemistry-based automated synthesis (Applied Biosystems, model 433A) and synthesis protocols based on previously published procedures.[27,28] The C-terminal lysine was introduced by using a MBHA polystyrene resin (NovaBiochem) pre-loaded with BOC-Lys(2-Cl-Z)-OH. Using a preactivation time of 5 min and a coupling time of 1 h, all novel monomers **10a**−**c** coupled with efficiencies comparable to the standard Boc-PNA monomers. The coupling efficiency was monitored by qualitative Kaiser test.[29] After cleavage and deprotection according to standard low/high TFMSA protocols, the PNA oligomers were purified by RP-C18 HPLC, analyzed by ESI-MS, lyophilized, and stored at −20 °C. HPLC buffer: A = 0.1% TFA in water; B = MeCN; 0 to 35% B in 35 min.

PNA monomer **10a** was incorporated into sequences **13** and **17**. Monomer **10b** was incorporated in sequences **14** and **18**, and monomer **10c** was incorporated into sequences **12** and **16**. All sequences are listed in Table 1. Sequence complementarity and stability of PNA-DNA and PNA-RNA hybrids were determined by thermal denaturation studies and

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Table 1. [30] T_m Data for Duplexes of 10-mer PNA Containing Cytosine (control), Phenoxazine (X = **2a**), G-clamp (Y = **2b**), and Propyl-G-clamp (Z = **2c**) with Complementary DNA and RNA

seq no	sequence	DNA		RNA	
		T_m °C	ΔT_m °K	T_m °C	ΔT_m °K
11	GTA GAT **C**AC T-Lys[20]	49.9		55.0	
12	GTA GAT **X**AC T-Lys	49.9	0.0	56.4	+1.4
13	GTA GAT **Y**AC T-Lys	[31]	--	65.7	+10.7
14	GTA GAT **Z**AC T-Lys	62.5	+13.5	68.5	+13.5
15	GTA GAT **C**CC T-Lys	49.8		60.1	
16	GTA GAT **C**XC T-Lys	55.6	+5.8	64.2	+4.1
17	GTA GAT **C**YC T-Lys	73.5	+23.7	78.3	+18.2
18	GTA GAT **C**ZC T-Lys	69.5	+19.7	76.1	+16.0

compared with the corresponding unmodified control sequences. The modifications were evaluated in two different sequences with the heterocylic modification at different positions within the oligomer and with different flanking bases. In the first set of oligomers (sequences **11**−**14**, Table 1), the flanking bases are T and A. In the second set (sequences **15**−**18**, Table 1) they are both C. While a single incorporation of the phenoxazine monomer (**10**c) in PNA **12** resulted in a T_m increase of only 1.4 °C when hybridized to complementary RNA, the PNA containing the G-clamp (PNA **13**) showed an affinity enhancement of 10.7 °C. PNA containing the propyl G-clamp, with the increased tether length at position 9 of the heterocycle (monomer **10b**, PNA **14**), resulted in further T_m advantage with an increase of 13.5 °C relative to unmodified PNA. When the heterocylic modification was flanked by two Cs in the second set of sequences, the T_m enhancement was even higher (Table 1). The phenoxazine modification (PNA **16**) resulted in a T_m increase of 4.1 °C toward complementary RNA. The G-clamp (PNA **17**) resulted in an increase of 18.2 °C. This T_m increase corresponds well to the data reported for a G-clamp-containing ODN of a similar sequence.[22] The enhancement of melting temperature for the G-clamp modification in ODNs also has significant sequence dependence.

Hybridization of the modified PNAs with complementary DNA gave results similar to those observed with complementary RNA (Table 1). The T_m enhancement was greater when the modified base was flanked by two Cs than when the flanking bases were T and A. A T_m enhancement of 23.7 °C was observed when the G-clamp PNA (PNA **17**) was hybridized to complementary DNA (Table 1). This T_m enhancement is the highest known increase in affinity for a PNA-DNA hybrid. The propyl G-clamp (PNA **18**) gave a T_m advantage of 19.7 °C when hybridized with complementary DNA.

For the control PNA[20] (**11**) and the phenoxazine modification (PNA **12**), incorporation of a base mismatch (A, T, or C) in the target strand opposite to the modified site resulted in noncooperative melting due to a lack of base specificity

Table 2. [30] Base Recognition of 10-mer PNA Containing Cytosine (control), Phenoxazine (X = **2a**), G-clamp (Y = **2b**), and Propyl-G-clamp (Z = **2c**) with Complementary G, A, C, and U of RNA

seq no	sequence	T_m °C			
		vs. rG	*vs.* rA	*vs.* rC	*vs.* U
11	GTA GAT **C**AC T-Lys[20]	55.0	[31]	[31]	[31]
12	GTA GAT **X**AC T-Lys	56.4	[31]	[31]	[31]
13	GTA GAT **Y**AC T-Lys	65.7	37.0	39.8	38.0
14	GTA GAT **Z**AC T-Lys	68.5	19.7	38.1	39.6

(Table 2). With G-clamp (PNA **13**) and propyl G-clamp (PNA **14**), significantly less stable duplexes were formed against oligonucleotides with mismatched A, C, and T as compared to the target strand containing a matching G (Table 2). The results indicate that all tricyclic modifications exhibit excellent sequence specificity.

In summary, tricyclic cytosine analogues based on the phenoxazine were incorporated into PNA oligomers. A convenient route for the facile synthesis of suitably protected phenoxazine, G-clamp, and propyl G-clamp PNA monomers has been developed. Single incorporation of these tricyclic moieties into to PNA showed sequence-dependent affinity enhancement of up to 18 °C when hybridized to RNA and up to 24 °C when hybridized to DNA. These are the highest T_m advantages for heterocyclic modifications of PNA reported so far. Furthermore, the magnitude depends on the length of the aminotether employed. Recently, we have also demonstrated the conversion of the G-clamp to the corresponding guanidinium group in oligonucleotide analogues and the same process can be extended to the PNAs reported here.[24,32] Further evaluation of these high-affinity modifications with respect to their effect on the cellular uptake and pharmacology of PNA antisense oligomers and on the strand invasion potential of PNA are in progress.

Acknowledgment. We thank our colleagues Drs. Ramesh Bharadwaj and Bruce S. Ross for 2-aminoresorcinol synthesis and Svetlana Doronina for earlier synthesis contributions in initiating this project. This work was supported in part by an SBIR grant (GM-60087).

Supporting Information Available: ESI-MS characterization of PNAs **11**−**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(30) The melting experiments were performed at 8 μM total strand concentration at pH 7.0 in 10 mM phosphate buffer containing 100 mM Na^+ and 0.1 mM EDTA. The T_m values presented are the average of three measurements and reproducibility of the measurements was within ±0.5 °C. [Lesnik, E. A.; Risen, L. M.; Driver, D. A.; Griffith, M. C.; Sprankle, K.; Freier, S. M. *Nucleic Acids Res.* **1997**, *25*, 568−574.]

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