Synthesis and Biophysical Studies of High Affinity Morpholino Oligomers Containing G-clamp Analogs

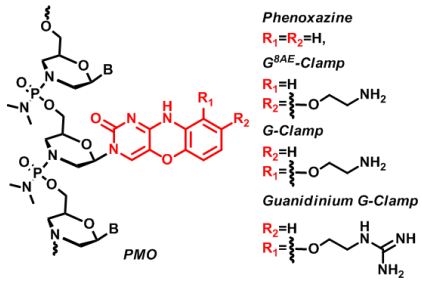
Arnab Das,[1] Atanu Ghosh,[1] Jayanta Kundu,[2] Martin Egli,[3] Muthiah Manoharan[2] and Surajit Sinha[1,*]

[1] School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700032, India

[2] Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States

[3] Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232, United States

*Corresponding author: ocss5@iacs.res.in



ABSTRACT: Synthesis of chlorophosphoramidate morpholino monomers containing tricyclic cytosine analogs phenoxazine, G-clamp, and G^{8AE}-clamp were achieved and incorporated into 12-mer oligonucleotides using trityl-chemistry by automated synthesizer. Phosphorodiamidate morpholino oligomers containing a single G-clamp had significantly enhanced affinity for complementary RNA and DNA relative to unmodified oligomers. The G-clamp-modified oligomers adopt a B-type helical conformation as per CD spectra. Binding affinities were sequence and position dependent.

For therapeutic use, nucleic acids must be chemically modified to increase affinity for complementary RNA strands and to improve nuclease resistance and cellular uptake.[1-5] The Watson-Crick base pairing of duplex formation is the foundation of biomolecular recognition. Increased affinity can be achieved by improving stacking interactions and/or hydrogen bonding.[6-10] Enhanced stacking can be accomplished by introducing polycyclic base analogs, and the number of H-bonds can be increased by engineering the simultaneous recognition of both the Watson-Crick and Hoogsteen binding faces of guanine and adenine bases. In a cytosine-guanine pair, the guanine has two unused H-bond acceptors in the major groove at the O6 and N7 atoms.[11,12] To form H-bonds with these acceptors, a tricyclic cytosine analog with an aminoethoxy-derivatized phenoxazine ring was designed by Matteucci (**Figure 1**).[11,12] This cytosine analog, referred to as the amino-G-clamp (G-clamp), was incorporated into oligonucleotides and shown to enhance duplex stability.[11-16] The proposed four H-bonds has been shown in **Figure S1**. The amino group of G-clamp has been converted to the guanidinium group which exhibits an unique five H-bonds base pair with opposite G utilizing O6 and N7 atoms and was confirmed by X-ray crystallography **(Figure S2).**[17,18]

In PNA, G-clamp modifications result the highest affinity for complementary DNA and RNA targets reported so far for PNA modifications.[19] G-clamps have also been used to modify 2′-O-methyl-modified RNA[20] and LNA.[21]

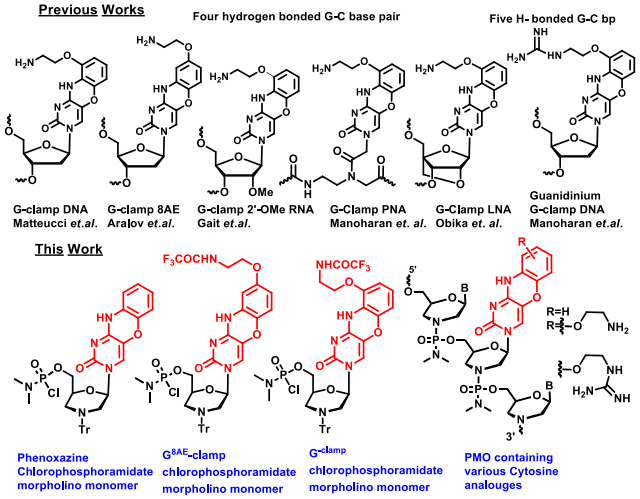
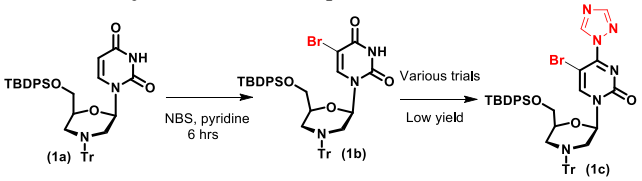


Figure 1: Previously reported G-clamps and chlorophosphoramidate morpholino monomers synthesized for this work.

Phosphorodiamidate Morpholino Oligomers (PMOs) are nucleic acid analogs based on morpholine rings joined by neutral phosphorodiamidate linkages. Developed by Summerton, PMOs have clinically proven therapeutic activity as splicing modulators.[22] In 2016, Eteplirsen became the first PMO approved for clinical use. It modulates splicing to treat Duchenne

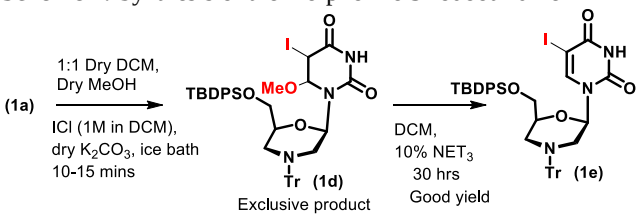
muscular dystrophy (DMD). Three additional PMO drugs are approved for the treatment of subtypes of DMD.[22b,c] As longer sequence of PMO is used for therapeutic applications than that of RNA, hence it's modification is required. For example, the antisense oligonucleotide Nusinersen is an 18-mer, whereas Eteplirsen is a 30-mer. Thus, modifications that enhance the binding affinity of PMO are highly desirable. To improve the binding affinity of PMOs, we prepared suitably protected amino- and guanidino-G-clamps, G^{8AE}-clamp, and phenoxazine morpholino (MO) monomers (**Figure 1**), and evaluated hybridization properties and other characteristics of PMOs containing these modifications.

Scheme 1: Synthesis of the morpholino 5-bromouridine



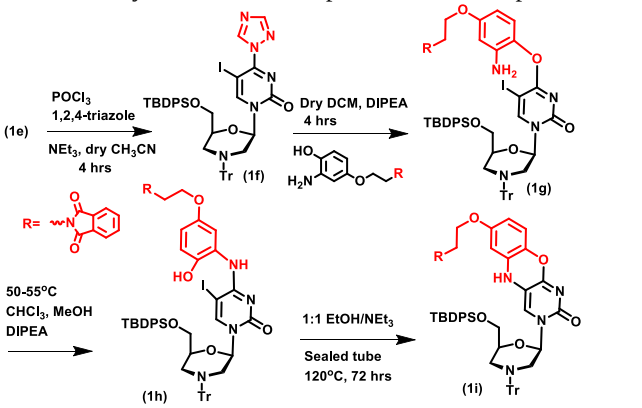
NBS/pyridine-mediated bromination of **1a**[23] yielded **1b** in high yield (**Scheme 1**). Since the conversion of **1c** from the bromo derivative **1b** was found to be poor (**Table S1**), we modified our approach by using the iodo derivative and synthesized (**Scheme 2**).

Scheme 2: Synthesis of the morpholino 5-iodoouridine



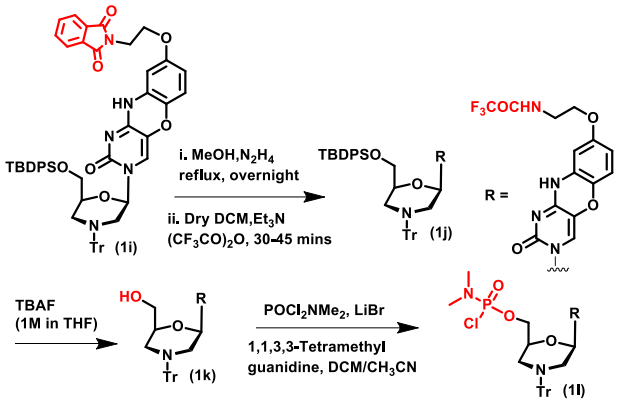
The C4 triazole **1f** was synthesized using $POCl_3$/1,2,4-triazole in good yield (**Scheme 3**). Treatment of **1f** with substituted aminohydroquinone (**Scheme S1**) gave **1g** as the major product. **1g** slowly isomerizes through a Smiles rearrangement to thermodynamically more stable **1h**, demonstrated by NMR spectroscopy (**SI**). The next challenge was the intramolecular cyclization of **1h.** After screening various conditions

Scheme 3: Synthesis of the morpholino G^{8AE}-clamp

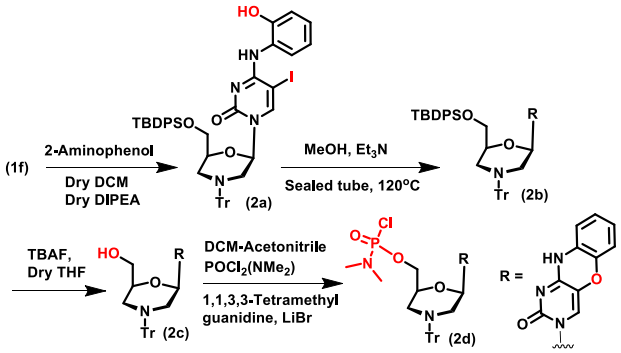


(**Table S2**), we found that heating **1h** in a sealed tube at 110 °C for 72hrs gave the desired **1i** in 36% yield. Phthalimide deprotection by hydrazine hydrate followed by protection of the free amine using trifluoroacetic anhydride (TFAA) gave **1j**. Tetrabutylammonium fluoride (TBAF)-mediated deprotection of the TBDPS group yielded **1k (Scheme 4)**. The chlororophosphoramidate monomer **1l** was synthesized using the LiBr-TMG method reported previously.[24] Phenoxazine derivatives (**2b-d**) were synthesized using a similar strategy (**Scheme 5**).

Scheme 4: Synthesis of the morpholino G^{8AE}-clamp chlorophosphoramidate monomer

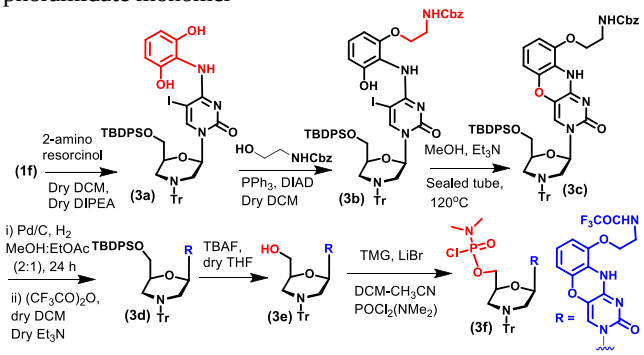


Scheme 5: Synthesis of the morpholino tricyclic phenoxazine chlorophosphoramidate monomer



To synthesize the morpholino G-clamp chlorophosphoramidate monomer **3f** (**Scheme 6**), **1f** was treated with 2-aminoresorcinol, yielding **3a**. *N*-phthalimide-protected ethanolamine was subjected to various Mitsunobu conditions, but the reaction failed. NH-Cbz-protected ethanolamine in presence of DIAD/PPh_3 in THF gave the mono-substituted product along with a very minute amount of deprotected product.

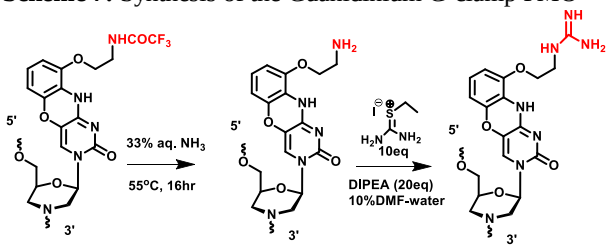
Scheme 6: Synthesis of the morpholino G-clamp chlorophosphoramidate monomer



3b was then cyclized to give **3c** as mentioned earlier for **2b**. Cbz was deprotected by 10% Pd/C under H_2 atmosphere. The free amine was purified and protected with trifluoroacetamide to obtain **3d**. After TBDPS deprotection, **3e** was converted to **3f** as discussed earlier.

The chlorophosphoramidate monomers were used in synthesis of two 12-mer PMOs (5′-TTTTACT**C**ACAT-3′ and 5′-TGT**C**ATC**C**CATT-3′, the bold letters are the sites of a single modification) on Ramage Chemmatrix resin following the reported procedure.[24,25] The PMOs were purified by HPLC and characterized by MALDI-TOF (**Table S3, Figure S3-S12**). A post-synthetic strategy was adopted for the conversion of amine to guanidinium group as previously reported.[26] After synthesis of PMO, the resin was treated with 33% aqueous NH_3 solution. The supernatant was lyophilized and treated with 2-ethylthiouronium iodide in presence of DIPEA in 10%-DMF/water at 55 °C for 24 h (**Scheme 7**) to get guanidinium PMO (**PMO-5** and **11)**.

Scheme 7: Synthesis of the Guanidinium G-clamp PMO



PMO number	Sequence	T_m with complementary DNA (ΔT_m) in °C	T_m with complementary RNA (ΔT_m) in °C
PMO-1[25]	5′-TTTTACTCACAT-3′	26	24
PMO-2	5′-TTTTACTXACAT-3′	30.0 (+4)	33.9 (+9.9)
PMO-3	5′-TTTTACTYACAT-3′	34.5 (+8.5)	39.4 (+15.4)
PMO-4	5′-TTTTACTZACAT-3′	36.5 (+10.5)	40.4 (+16.4)
PMO-5	5′-TTTTACTWACAT-3′	40.6 (+14.6)	48.4 (+24.4)
PMO-6	5′-TGTCATCCCATT-3′	41.2	50.3
PMO-7	5′-TGTXATCCCATT-3′	39.2 (-2)	46.5 (-3.8)
PMO-8	5′-TGTYATCCCATT-3′	47.6 (+6.4)	60.2 (+9.9)
PMO-9	5′-TGTZATCCCATT-3′	54.2 (+13)	66.9 (+16.6)
PMO-10	5′-TGTCATCZCATT-3′	60.2 (+19)	69.0 (+18.7)
PMO-11	5′-TGTCATCWCATT-3′	56.6 (+15.4)	63.8 (+13.5)

Table 1: T_m of modified PMOs with complementary DNA and RNA. X = **2d**, phenoxazine, Y = **1l**, G^{8AE}-clamp, Z =**3f**, G-clamp, W=guanidino G-clamp. Conditions: 40 mM phosphate buffer (pH 7). The concentration of each strand was 1µM. The T_m values reported are the averages of two independent experiments and results differed by less than ±1.0°. The ΔT_m values are in comparison to unmodified PMO-1 or PMO-6.

Inflection point from the first derivative plot of thermal melting curves (T_ms) were determined for the modified and unmodified 12-mer PMOs with complementary DNA and RNA (**Table 1**, **Figure S13-S22**). Incorporation of phenoxazine (**X)** in **PMO-2** greatly enhanced the RNA-PMO duplex stability in compare to the duplex of unmodified PMO; however, slight destabilization was observed in the case of **PMO-7**. The phenoxazine is hydrophobic, may have unfavorable dehydration effects on the groove during duplex formation in certain sequence contexts. T_m values for the phenoxazine and its analogs were previously reported to be sequence dependent.[15] Incorporation of G^{8AE}-clamp **Y** (**PMO-3** and **PMO-8**) increased the T_m values relative to the unmodified PMO by between +15.4 and +9.9°C. A model of the Y:G pair shows that the AE amino group of Y can approach the O6 atom of the paired G (ca. 3.5 Å; **Figure 2**). In this model, it is slightly outside the distance range that would allow effective H-bond formation. However, the introduction of a positively charged moiety into the center of the major groove, a site of strong negative electrostatic surface potential (ESP), and in vicinity of the O6/N7 edge of G, still affords a stabilizing effect as per the T_m data. Moreover, incorporation of G-clamp and G^{8AE}-clamp may produce stability-enhancing effects that differ from those seen for the guanidino G-clamp (H-bonds to O6 and N7 of the paired G, **Figure S1A**).[17,18] Thus, the G- and G^{8AE}-clamp amino group could also scan the major groove edges of bases from the 5′- and 3′-adjacent intra-strand residues (**Figure S2**). By comparison, the guanidino moiety of the guanidino G-clamp is lodged exclusively opposite the major groove edge of the paired G (**Figure S1A**). This promiscuity of the (amino) G-clamp may be an underappreciated feature that distinguishes it from the guanidino G-clamp. The term clamp may be more appropriate for the latter, whereas the original G-clamp could boost stability in a sequence-dependent manner: H-bonding to the paired G, the major groove edges of bases one step up or down, and inserting a positive charge into a region of negative ESP. This could explain the previous observation whereby the G-clamp typically results in higher T_m gains than the guanidino G-clamp. This is confirmed here by the thermal melting data for PMO-modified duplexes. Thus, incorporation of the G-clamp **Z** increased the T_m by 16.4 °C and 16.6 °C (**PMO-4** and **PMO-9**, respectively). **PMO-10**, in which the G-clamp is located between two cytosines, showed much higher duplex stabilities with DNA and RNA than **PMO-9**. Interestingly, the guanidino G-clamp **W** was stabilizing in the case of **PMO-5** but not in case of **PMO-11.**

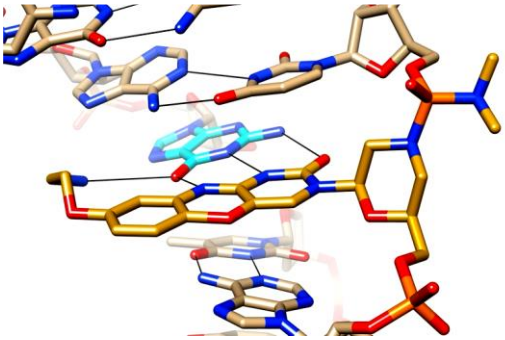


Figure 2: Model of PMO G^{8AE}-clamp Y:G pairing. The AE amino nitrogen is positioned at ca. 3.5 Å from O6 of G. PMO and guanine carbon atoms are highlighted in golden rod and cyan, respectively, and H-bonds are shown as thin solid lines.

The global conformations of the duplexes of PMOs with DNA and RNA were evaluated by CD spectra at 10 °C. All duplexes with RNA had absorption maxima at approximately 270 nm and 220 nm with sharp minima at about 245 nm (**Figure 3A, B**). Similar spectra were observed for duplexes with DNA (**Figure S23-24**). These spectra are typical of a B-type helical conformation for both DNA and RNA complements.

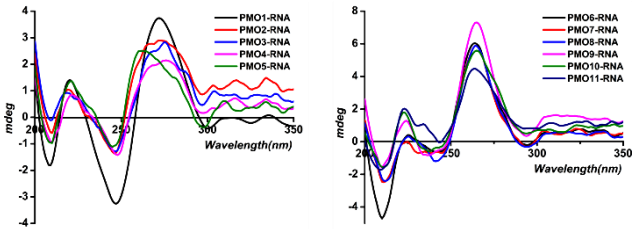


Figure 3: CD-spectra of **(A)** duplexes of PMO-1-5 with RNA, **(B)** duplexes of PMO-6-11 with RNA,

In summary, a convenient synthetic methodology was developed for the phenoxazine and its derivatives, the G^{8AE}-clamp and the G-clamp. The newly synthesized phenoxazine, G^{8AE}-clamp, G-clamp, and guanidino G-clamp PMO cytidines were incorporated into PMOs. Duplexes of PMOs with single modifications had higher thermal stabilities with the complementary DNA and RNA than the unmodified PMO and indicated the B-type helical structure. The aminoethoxy G-clamp tethered to the C8 and C9 of the tricyclic nucleobases show different melting behavior as expected due to the relative H-bonding abilities. The thermal stabilities of the duplexes were sequence dependent. The guanidino G-clamp also stabilizes the duplex but the extent of stabilization varies depending upon the nature and position. Given the enhanced affinities of the G-clamp-modified PMO for RNA, this modification could allow development of shorter PMOs than those currently in clinical use for splice modulation or might improve the potency of PMOs with the same length.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and characterization data including the spectra for all new compounds can be found in the supporting information.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

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