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Synthesis of Conformationally Preorganized and Cell-Permeable Guanidine-Based γ-Peptide Nucleic Acids (γGPNAs)

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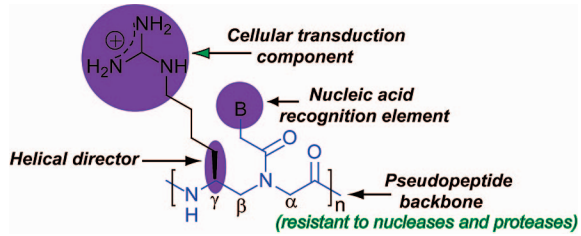

A general method for preparing optically pure guanidine-based γ-peptide nucleic acid (γGPNA) monomers for all four natural nucleobases (A, C, G, and T) is described. These second-generation γGPNAs differ from the first-generation GPNAs in that the guanidinium group is installed at the γ- instead of the α-position of the *N*-(2-aminoethyl)glycine backbone unit. This positional switch enables GPNAs to be synthesized from relatively cheap L- as opposed to D-amino acids. Unlike their α-predecessors, which are randomly folded, γGPNAs prepared from L-amino acids are preorganized into a right-handed helix and bind to DNA and RNA with exceptionally high affinity and sequence selectivity and are readily taken up by mammalian cells.

Introduction

One of the major challenges facing antisense and antigene technology is cellular delivery. Several classes of oligonucleotides have been developed that can recognize and bind to DNA and RNA with high affinity and sequence selectivity and withstand enzymatic degradation by proteases and nucleases; however, few can traverse the cell membrane on their own.[1−4] Additional chemical, mechanical and/or electrical transduction means are generally employed to transport these oligonucleotides into cells.[5,6] While this may be a viable option for small-scale experimental setups, it is not practical for many *in vivo* experiments or therapeutic and diagnostic applications. Aside from the technical challenge, the use of such exogenous transduction reagents/means can lead to off-target and cytotoxic effects.[7] It is therefore essential to be able to modify the structures and/or chemical functionalities of these oligonucleotides further so that their cellular uptake and other desired features can be augmented and/or improved upon without the need for auxiliary transduction reagents/means.

Peptide nucleic acid (PNA) is a particularly promising class of nucleic acid mimics, developed in the last decade, in which the natural sugar-phosphodiester backbone has been replaced by the achiral *N*-(2-aminoethyl)glycine units.[8] The neutral

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(1) De Mesmaeker, A.; Haener, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366–374.
(2) Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301–310.
(3) Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624–630.
(4) Summerton, J. E. *Lett. Pept. Sci.* **2004**, *10*, 215–236.
(5) Juliano, R. L.; Yoon, H. *Curr. Opin. Mol. Therap.* **2000**, *2*, 297–303.
(6) Lebedeva, I.; Benimetskaya, L.; Stein, C. A.; Vilenchik, M. *Eur. J. Pharm. Biopharm* **2000**, *50*, 101–119.
(7) Stein, C. A. *Nat. Biotechnol.* **1999**, *17*, 209.
(8) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497–1500.

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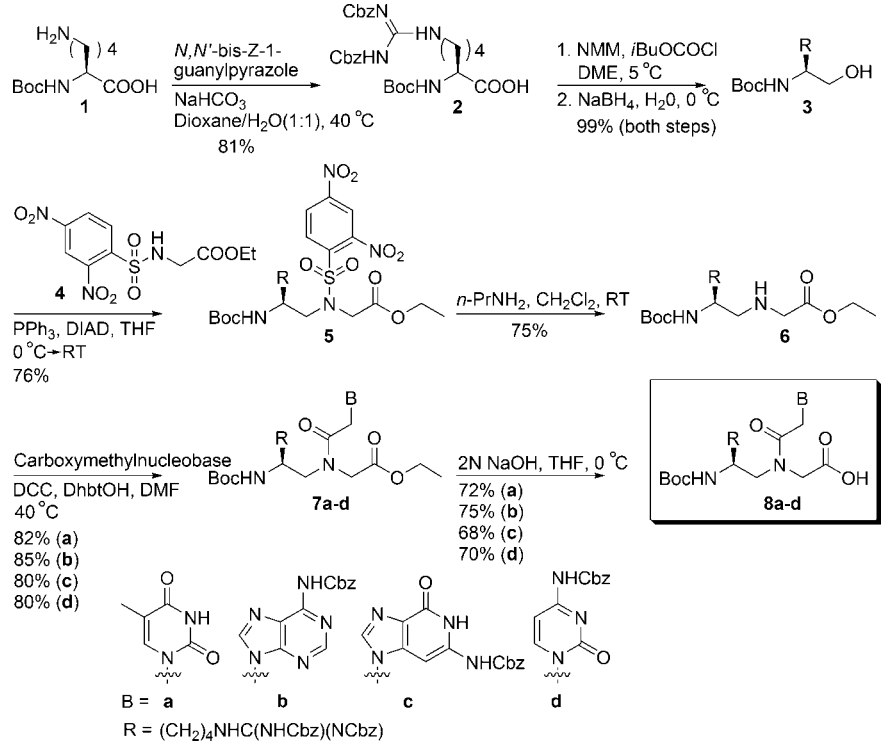
SCHEME 1. Synthesis of γGPNA Monomers



backbone allows PNA to hybridize to DNA and RNA with high affinity (and sequence selectivity) through Watson−Crick base-pairing, while the unnatural polyamide linkage allows PNA to withstand enzymatic degradation by proteases and nucleases.[3] These properties, along with the ease of its synthesis, have made PNA an attractive reagent for many applications in chemistry, biology and medicine.[9] However, the full potential of PNA has not yet been fully realized due, in large part, to its poor cellular uptake properties[10]—although some progress has been made in recent years.[11]

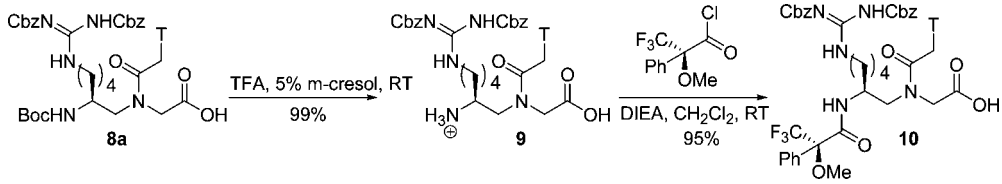
Inspired by the facile uptake of HIV-1 TAT protein,[12,13] and related peptides[14−16] and peptidomimetics,[17,18] we have recently shown that covalent attachment of arginine side-chain to the α-position of *N*-(2-aminoethyl)glycine backbone unit enables PNAs (GPNAs) to get into cells.[19−21] This design differs from the conventional conjugation approach in that the recognition and transduction elements are integrated into a single unit. This strategy requires fewer coupling steps in the oligomer synthesis and minimizes the amphiphatic character of the oligonucleotides, making them less disruptive to the cell membrane and therefore less toxic to the cells.[22] The ability of these oligomers to traverse the cell membrane and bind to their designated RNA targets has already been demonstrated in our recent studies, in which we showed potent and selective inhibition of E-cadherin gene expression in mammalian cells[23] and in the systemic delivery and treatment of squamous cell carcinoma (SCCHN) xenograph model (data not shown). Although these results are promising, GPNAs cannot be prepared in large scale for clinical testing because of the high cost associated with monomer synthesis. They need to be prepared from relatively expensive D-amino acids because the L-configuration does not allow them to bind effectively to DNA or RNA.[21] In this Article, we report the synthesis of the second-generation GPNAs, which can be prepared from relatively cheap L-amino acids and exhibit favorable hybridization and cellular uptake properties. As single strands, these γGPNA oligomers are already preorganized into a right-handed helix and bind to DNA and RNA with high affinity and sequence selectivity and are readily taken up by mammalian cells.

Results and Discussion

Monomer Synthesis. The γGPNA monomers for all four natural nucleobases (A, C, G and T) were prepared according to the procedures outlined in Scheme 1. Boc-L-Lysine was selected as a starting material for the synthesis over Boc-L-ornithine and suitably protected Boc-L-Arg(di-Z), which are also commercially available, based purely on cost—although Wender and co-workers[17] showed that an extension in the side-chain

(9) Nielsen, P. E. *Mol. Biotech.* **2004**, *26*, 233–248.
(10) Nielsen, P. E. *Q. Rev. Biophys.* **2005**, *38*, 345–350.
(11) Koppelhus, U.; Nielsen, P. E. *Adv. Drug Deliv. Rev.* **2003**, *55*, 267–280.
(12) Frankel, A. D.; Pabo, C. O. *Cell* **1988**, *55*, 1189–1193.
(13) Green, M.; Loewenstein, P. M. *Cell* **1988**, *55*, 1179–1188.
(14) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. *Science* **1999**, *285*, 1569–1572.
(15) Gratton, J.-P.; Yu, J.; Griffith, J. W.; Babbitt, R. W.; Scotland, R. S.; Hickey, R.; Giordano, F. J.; Sessa, W. C. *Nat. Med.* **2003**, *9*, 357–362.
(16) Lewin, M.; Carlesso, N.; Tung, C.-H.; Tang, X.-W.; Cory, D.; Scadden, D. T.; Weissleder, R. *Nat. Biotechnol.* **2000**, *18*, 410–414.
(17) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003–13008.
(18) Umezawa, N.; Gelman, M. A.; Haigis, M. C.; Raines, R. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 368–369.
(19) Zhou, P.; Wang, M.; Du, L.; Fisher, G. W.; Waggoner, A.; Ly, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 6878–6879.
(20) Zhou, P.; Dragulescu-Andrasi, A.; Bhattacharya, B.; O'keefe, H.; Vatta, P.; Hyldig-Nielsen, J. J.; Ly, D. H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4931–4935.
(21) Dragulescu-Andrasi, A.; Zhou, P.; He, G.; Ly, D. H. *Chem. Comm.* **2005**, 244–246.
(22) Zasloff, M. *Nature* **2002**, *415*, 389–395.
(23) Dragulescu-Andrasi, A.; Rapireddy, S.; He, G.; Bhattacharya, B.; Hyldig-Nielsen, J. J.; Zon, G.; Ly, D. H. *J. Am. Chem. Soc.* **2006**, *128*, 16104–16112.

SCHEME 2. Derivatization of γGPNA Monomer for ^{19}F-NMR Analysis



length can have a beneficial effect on cellular uptake. Boc-L-lysine was first functionalized with *N*,*N*′-(*Z*)-1-guanylpyrazole. Reduction of the activated ester of intermediate (**2**) with $NaBH_4$, followed by Mitsunobu coupling with 2-(2,4-dinitrophenylsulfonamido)acetate (**4**) afforded a stable, fully protected backbone (**5**) that can be stored over a prolonged period. We selected Mitsunobu reaction over reductive amination, a method previously used to prepare other γPNAs,[24−29] because of the enantioselective nature of this coupling reaction.[30] Instead of generating an aminoaldehyde, which is known to undergo epimerization,[31,32] the amino acid is converted into an aminoalcohol (**3**) in the second step. This chemical transformation renders the α-proton inert to deprotonation by the base, which is the source of racemization. Removal of the dinitrosulfonate protecting group, followed by standard DCC-mediated coupling with the carboxymethylnucleobase[33] and hydrolysis yielded the desired monomers (**8a**−**d**).

Enantiomeric Excess (ee) Determination. The optical purities of the monomers were determined by ^{19}F-NMR. Prior to analysis, the Boc-protecting group was first removed with TFA (with 5% m-cresol) and then coupled with chiral shift reagent, (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) (Scheme 2).[34] Figure 1 shows the representative ^{19}F-NMR spectra of thymine (T) γGPNA monomers prepared from Boc-protected L- and D-lysine following identical reaction conditions. No trace quantity of racemic mixture was observed within the detection limit of ^{19}F-NMR, which was determined to be ∼0.5% ee.

To determine the effect of γ-backbone modification on the conformations and hybridization properties of PNAs, we synthesized a series of oligomers where the new building blocks were strategically incorporated with varying number and spatial arrangements (Table 1) and characterized their properties by CD and UV−vis spectroscopic techniques. The oligomers were synthesized manually on MBHA resins according to standard procedures.[35] After cleavage and removal of the protecting groups, the oligomers were purified to greater than 95% homogeneity by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry.

TABLE 1. Sequence of the Oligomers Used in This Study along with Their MW

oligomers	sequence[a]	cal. MW (found)	% yield[b]
PNA1	H-GCATGTTTGA-^{L}Lys-NH_2	2886.8 (2885.8)	35
γGPNA1	H-GCATG$\underline{T}$TTGA-NH_2	2869.1 (2874.3)	32
γGPNA2	H-GCATG$\underline{TT}$TGA-NH_2	2982.2 (2991.2)	37
γGPNA3	H-GCATG$\underline{TTT}$GA-NH_2	3095.3 (3098.5)	33
γGPNA4	H-GCA$\underline{T}$G$\underline{TT}$G$\underline{T}$GA-NH_2	3095.3 (3098.9)	27
γGPNA5	H-G$\underline{CA}$G$\underline{TT}$G$\underline{TT}$G$\underline{A}$-NH_2	3321.5 (3325.8)	31
γGPNA6	TAMRA-G$\underline{CA}$G$\underline{TT}$G$\underline{TT}$G$\underline{A}$-NH_2	3825.7 (3824.6)	29
PNA2	TAMRA-GCATGTTTGA-^{L}Lys-NH_2	3355.2 (3378.8)	33
TAT	TAMRA-YGRKKRRQRRR-NH_2	1972.2 (1973.2)	40

[a] Underlined sequence denotes γGPNA backbone modification. [b] After purification.

Conformational Analysis. CD measurements were performed for PNA (PNA1) and γGPNA (γGPNA1 through 5) oligomers at 5 μM strand concentration each in 10 mM sodium phosphate buffer (pH 7.4) at room temperature (Figure 2). No CD signals were observed for PNA1 in the nucleobase absorption regions (220−320 nm). This result indicates one of two possibilities—either that it does not adopt helical information (absence of base-stacking), or that it does adopt helical conformation but with an equal proportion of a right- and left-handed helix, as suggested by MD simulations.[36] We ruled out the second possibility based on 2D-NMR analysis.[37] In addition to the lack of helical preorganization, unhybridized PNAs have the tendency to fold into globular structures due to solvophobic effect. This phenomenon has been exploited in the development of "stemless" PNA molecular beacons, taking advantage of the proximity of the two termini in the unhybridized state.[38−40] However, in the case of γGPNA1 through 5, we observed distinct CD signals, with the characteristic pattern of a right-handed helix[41,42]—with maxima at 220 and 260 nm, and minima at 245 and 290 nm. The helical sense of this particular class of γPNAs has been confirmed by 2D-NMR.[37] The amplitudes of the CD signals gradually increased with increasing number of γGPNA units in the oligomers, showing improvements in base-stacking. Between γGPNA3 and γGPNA4, both with three γ-modified backbone units, γGPNA4, which contained alternate backbone spacing, exhibited greater CD signals than γGPNA3, which contained consecutive backbone spacing. This result is

(24) Kosynkina, L.; Wang, W.; Liang, T. C. *Tetrahedron Lett.* **1994**, *35*, 5173–5176.
(25) Wu, Y.; Xu, J. C. *Tetrahedron* **2001**, *57*, 8107–8113.
(26) Englund, E. A.; Appella, D. H. *Org. Lett.* **2005**, *7*, 3465–3467.
(27) Tedeschi, T.; Sforza, S.; Corradini, R.; Marchelli, R. *Tetrahedron Lett.* **2005**, *46*, 8395–8399.
(28) Dose, C.; Seitz, O. *Org. Lett.* **2005**, *7*, 4365–4368.
(29) Englund, E. A.; Appella, D. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1414–1418.
(30) Falkiewicz, B.; Kolodziejczyk, A. S.; Liberek, B.; Wisniewski, K. *Tetrahedron* **2001**, *57*, 7909–7917.
(31) Stanfield, C. F.; Parker, J. E.; Kanellis, P. *J. Org. Chem.* **1981**, *46*, 4797–4798.
(32) Rittle, K. E.; Homnick, C. F.; Ponticello, G. S.; Evans, B. E. *J. Org. Chem.* **1982**, *47*, 3016–3018.
(33) Dueholm, K. L.; Egholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K. H.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Org. Chem.* **1994**, *59*, 5767–5773.
(34) Seco, J. M.; Quinoa, E.; Riguera, R. *Chem. Rev.* **2004**, *104*, 17–117.
(35) Christensen, L.; Fitzpatrick, R.; Gildea, B.; Petersen, K. H.; Hansen, H. F.; Koch, T.; Egholm, M.; Buchardt, O.; Nielsen, P. E.; Coull, J.; Berg, R. H. *J. Pept. Sci.* **1995**, *1*, 175–183.
(36) Sen, S.; Nilsson, L. *J. Am. Chem. Soc.* **2001**, *123*, 7414–7422.
(37) Dragulescu-Andrasi, A.; Rapireddy, S.; Frezza, B. M.; Gayathri, C.; Gil, R. R.; Ly, D. H. *J. Am. Chem. Soc.* **2006**, *128*, 10258–10267.
(38) Seitz, O. *Angew. Chem., Int. Ed.* **2000**, *39*, 3249–3252.
(39) Ranasinghe, R. T.; Brown, T. J.; Brown, L. J. *Chem. Comm.* **2001**, 1480–1481.
(40) Kuhn, H.; Demidov, V. V.; Coull, J. M.; Fiandaca, M. J.; Gildea, B. D.; Frank-Kameneteskii, M. D. *J. Am. Chem. Soc.* **2002**, *124*, 1097–1103.
(41) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566–568.
(42) Wittung, P.; Nielsen, P. E.; Buchardt, O.; Egholm, M.; Norden, B. *Nature* **1994**, *368*, 561–563.

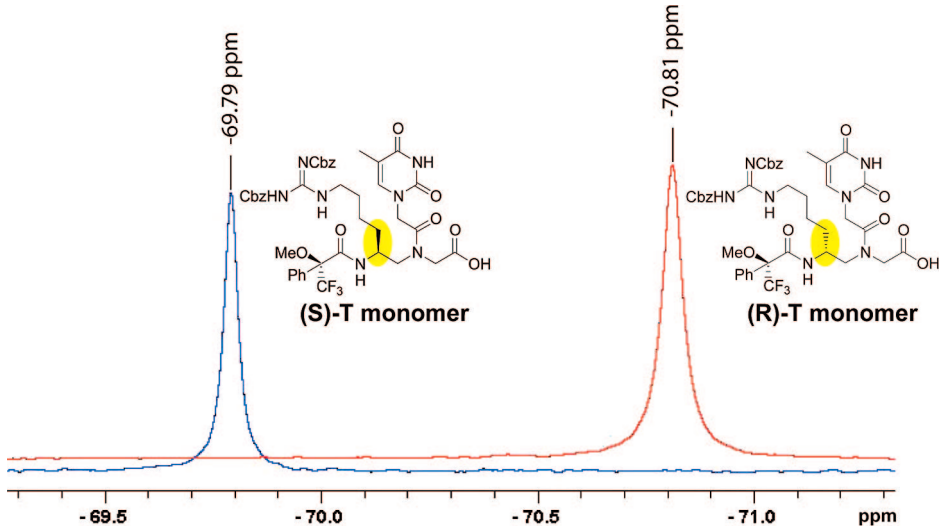


FIGURE 1. ^{19}F-NMR spectra of thymine-containing γGPNA monomers prepared from L-(left) and D-lysine (right) following the removal of Boc-protecting group and coupling with MTPA-Cl.

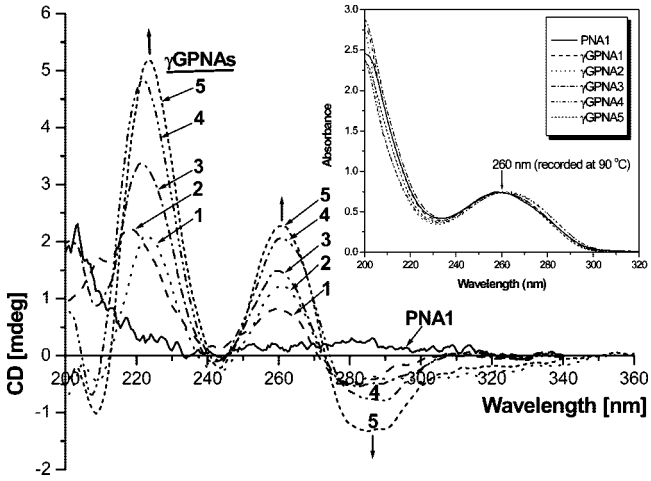


FIGURE 2. CD spectra of individual (single-stranded) PNA and γGPNA oligomers. All samples were prepared in buffer containing 10 mM sodium phosphate, 100 mM NaCl and 0.1 mM EDTA at 5 μM strand concentration each. (Inset) UV-absorbance of each sample recorded at 90 °C showing that the amount of PNA and γGPNA in each sample was virtually the same.

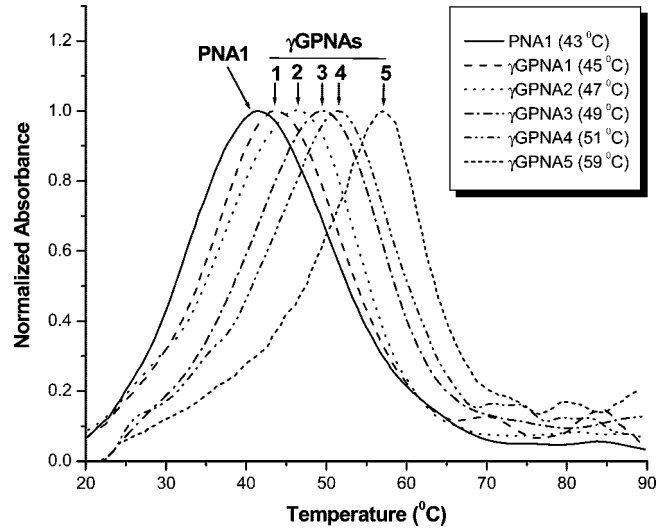


FIGURE 3. First-derivatives of the PNA-DNA and γGPNA-DNA UV-melting profiles. The hybrid duplexes were prepared by mixing stoichiometric amount (2 μM strand concentration) of each oligomer in the same buffer used in CD measurements and annealed by incubating at 90 °C for 5 min, followed by gradual cooling to room temperature. UV-absorbance at 260 nm was monitored as a function of temperature from 20 to 90 °C at the rate of 1 °C/minute.

consistent with our unpublished finding which showed that nucleobases are stacked better in the alternate than in the consecutive arrangement, consistent with the "Sergeant and Soldiers" concept proposed by Green and co-workers[43,44] to explain helical induction in polymers.

Hybridization Properties. Next, we assessed the hybridization properties of PNA and γGPNA oligomers following their hybridization with a complementary (antiparallel) DNA strand. UV-melting analysis of the corresponding duplexes revealed notable improvements in the binding affinity, on average, by ~2 °C for every γ-modified backbone unit incorporated (Figure 3). This level of stabilization is significant compared to the first-generation α-GPNAs, which showed only slight improvements when they were prepared from D-amino acids with positively charged side-chains, such as lysine or arginine,[21] while the rest, L-amino acids in particular, had deleterious effects on the binding properties of PNA.[45] Alternate backbone spacing showed a slight enhancement in binding affinity as compared to the consecutive arrangement (compare the T_m of γGPNA4 to that of γGPNA3 in Figure 3). This result is consistent with the CD data which showed that the nucleobases in γGPNA4 are better stacked with one another than those in γGPNA3, as apparent from the amplitude of the CD signals. We attribute the enhancement in thermal stability of γGPNA-DNA duplex, in large part, to conformational preorganization and not electrostatic interactions between guanidine and phosphate groups since PNA oligomers with (γGPNA5) and without (PNA1) the guanidinium groups exhibited similar salt-dependent melting profiles (Figure S1, Supporting Information). MD

(43) Green, M. M.; Peterson, N. C.; Sato, T.; Teramoto, A.; Cook, R.; Lifson, S. *Science* **1995**, *268*, 1860–1866.
(44) Green, M. M.; Cheon, K.-S.; Yang, S.-Y.; Park, J.-W.; Swansburg, S.; Liu, W. *Acc. Chem. Res.* **2001**, *34*, 672–680.
(45) Haaima, G.; Lohse, A.; Buchardt, O.; Nielsen, P. E. *Angew. Chem., Int. Ed.* **1996**, *35*, 1939–1941.

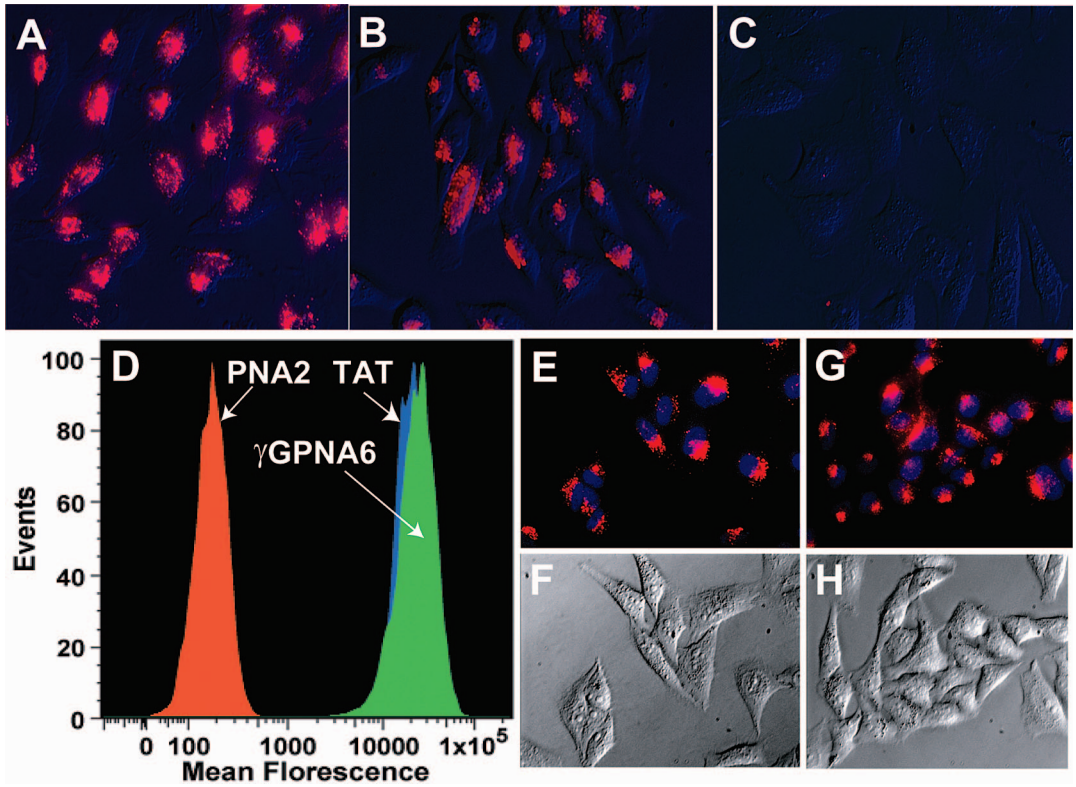


FIGURE 4. (A−C) Superimposed DIC and fluorescent images of HeLa cells after incubation with 1 μM of γGPNA6, TAT and PNA2, respectively, for 24 h. Cells were washed 3 times with PBS and immediately imaged (these were live cells). (D) FACS analysis of HeLa cells following incubation with the same oligomer concentration and period (20 000 cells were taken for the events). (E and G) Fluorescent images of HeLa cells incubated with 1 μM of γGPNA6 and TAT, respectively, for 24 h, followed by brief washing with PBS and incubation with Hoechst (1 mg/mL) in fresh growth media for 5 min. Blue: nucleus (Hoechst), red: oligomers (TAMRA). (F and H) Bright field images of panels E and G, respectively.

TABLE 2. Sequence Discrimination of PNA1 and γGPNA5 for DNA and RNA[a]

H-GCATGTTTGA-^{L}Lys-NH_2 PNA1
H-GCATGTTTGA-NH_2 γGPNA5
3'-CGTACXAACT-5' DNAX
3'-CGUACXAACU-5' RNAX

	PNA1-DNAX	γGPNA5-DNAX		PNA1-RNAX	γGPNA5-RNAX
X = A, PM	43 °C	59 °C	X = A, PM	50 °C	60 °C
X = C, T <> C MM	25 (−18)	39 (−20)	X = C, T <> C MM	33 (−17)	40 (−20)
X = G, T <> G MM	25 (−18)	40 (−19)	X = G, T <> G MM	40 (−10)	48 (−12)
X = T, T <> T MM	32 (−11)	40 (−19)	X = U, T <> U MM	39 (−11)	49 (−11)

[a] PM: perfect match, MM: mismatch. The duplex concentration is 2μM prepared in the same buffer used in CD measurements.

simulations revealed that the lysine side-chain is too short for the guanidinium groups on γGPNA to reach across the double helix and interact with the phosphates on the opposing DNA backbone (unpublished data).

We selected γGPNA5 for further analysis because it contained a minimum number of guanidine groups needed to traverse the cell membrane.[21] Inspection of Table 2 reveals that γGPNA5 binds to (antiparallel) complementary DNA and RNA strand with significantly higher affinity than the unmodified PNA1 (+16 and +10 °C for the DNA and RNA, respectively). It is interesting to note that the T_ms of the perfectly matched γGPNA5-DNA and γGPNA5-RNA are nearly identical. In the case of unmodified PNA, a PNA-RNA is generally more thermodynamically stable than a PNA-DNA duplex.[41,46] This result indicates that γGPNAs are just as accommodating to RNA as they are to DNA. The sequence-mismatch selectivities of γGPNA5 for DNA and RNA are similar to that of PNA1 (Table 2). Our data show that the positively charged guanidinium groups in the backbone of PNAs have little influence on their sequence selectivity.

Cellular Uptake. Next, we determined the uptake properties of γGPNA6 (γGPNA5 with TAMRA covalently attached at the N-terminus) and compared to that of TAT transduction domain and unmodified PNA (PNA2). HeLa cells in logarithmic phase cultured in complete media were incubated separately with 1 μM of γGPNA6, TAT and PNA2. After 24 h of incubation, cells were briefly washed three times with PBS buffer and immediately imaged without fixing. Selective uptake of γGPNA6 and TAT was observed (Figure 4A and B), as evidenced from the fluorescent signals, while none was detected for PNA2

(46) Jensen, K. K.; Orum, H.; Nielsen, P. E.; Norden, B. *Biochemistry* **1997**, *36*, 5072–5077.

(Figure 4C). FACS analysis of the same cell population following identical treatment (1 μM oligomer concentration and 24 h incubation) reveals similar uptake efficiency for γGPNA6 and TAT, which is about 2 orders of magnitude higher than that for PNA2 (Figure 4D).

In a second set of experiment, live cells were incubated with the aformentioned oligomers for 24 h. Following brief washing with PBS, nuclear staining dye (Hoechst-33258) was added and cells were imaged after 5-min incubation. For both γGPNA6 (Figure 4E and F) and TAT (Figure 4G and H), the fluorescent signals appeared to localize within the vicinity of the nucleus with similar intensity—most likely in the endoplasmic reticulum (ER), where protein biosynthesis takes place. This result is similar to the observation made with the first-generation α-GPNAs.[20] At this point it is not clear why these cationic oligomers appear to localize specifically at the ER. One possibility is that γGPNAs are trapped within the ER because of the electrostatic and/or nucleobase interactions between γGPNAs and RNA molecules—after all, this is where most of the RNA molecules reside following their export from the nucleus. Although the cause of this ER-specific localization is still presently unknown, our result shows that the fully alternate γGPNA oligomer is taken up by HeLa cells just as efficient as TAT transduction domain. The fact that γGPNAs, just like TAT domain, are localized in the ER makes them attractive reagents for antisense applications.

Conclusion

In summary, we have shown that optically pure γGPNA monomers could be prepared from relatively cheap Boc-L-lysine by employing Mitsunobu coupling reaction. γGPNA oligomers, unlike their aminoethylglycyl counterparts which do not have well-defined conformations, are already preorganized into a right-handed helix. These oligomers hybridize with DNA and RNA with high affinity and sequence selectivity, and are readily taken by mammalian cells. The improved cellular uptake of γGPNAs could be attributed to their helical conformation, which has been shown to facilitate cellular delivery in other systems.[47,48] Molecules that can be programmed to bind selectively to DNA and RNA, and are taken up by cells are valuable for many applications in biology and medicine. Such molecules could be used as molecular tools to probe and manipulate nucleic acid structures and functions in cell cultures and in intact organisms, as well as therapeutic and diagnostic reagents for the treatment and detection of genetic diseases, such as cancers. Also, because of the synthetic flexibility at the γ-position, other functional groups can be incorporated in the backbone of PNA. This makes it an attractive platform for launching nucleic acid–based therapeutics and diagnostics.

Experimental Section

Boc-Lys (Guanidine-Di-Cbz)-OH (2). To a stirred solution of $NaHCO_3$ (2.13 g, 25.37 mmol) in dioxane: water (1:1, 80 mL) was added Boc-Lys-OH (5 g, 20.3 mmol), followed by *N*,*N*′-bis-(*Z*)-1-guanylpyrazole (5.74 g, 15.2 mmol). The resulting mixture was stirred at 40 °C for 48 h. The solvent was removed under reduced pressure. The resulting residue was dissolved in ethyl acetate, washed with diluted HCl (0.3 M) and then brine, and dried over Na_2SO_4 (anhyd). The solvent was evaporated *in vacuo* and the resulting residue was purified by column chromatography and dried to give a colorless solid (9.2 g, 16.5 mmol); yield 81%. ^{1}H NMR ($CDCl_3$) δ 8.45−8.22 (br s, 1H), 7.50−7.35 (m, 10 H), 5.20−5.15 (s, 2H), 5.11−5.00 (s, 2H), 4.40−4.20 (m, 1H), 3.50−3.30 (m, 2H), 2.00−1.60 (m, 4H), 1.50−1.35 (m, 11 H); ^{13}C NMR ($CDCl_3$) δ 22.6, 28.3, 28.5, 32.1, 40.8, 53.2, 67.1, 68.2, 80.0, 127.9, 128.0, 128.4, 128.7, 128.8, 136.7, 153.8, 156.0, 163.6, 176.0; LRMS: 557.20; HRMS: (ESI-MS *m*/*z*) M_{calc} for $C_{28}H_{37}N_4O_8$ 557.2612, found 557.2656.

Boc-Lys (Guanidine-Di-Cbz)-ol (3). Boc-Lys (guanidine-di-cbz)-OH (**2**) (5 g, 8.9 mmol) was dissolved in 10 mL DME and cooled in an ice bath. To this mixture was added NMM (0.97 mL, 8.9 mmol) dropwise, followed by isobutyl-chloroformate (1.2 mL, 8.9 mmol). The reaction mixture was stirred in the ice-bath for another 0.5 h. The solution was filtered, and the precipitate was washed with DME (2 × 10 mL). To the filtrate, $NaBH_4$ (0.505 g, 13.3 mmol, in 10 mL water) was added at 0 °C. The aqueous layer was extracted several times with ethyl acetate and the combined organic layers were washed with brine, dried over Na_2SO_4 (anhyd). The solvent was evaporated *in vacuo* to obtain a sticky liquid. The crude product was purified by column chromatography and dried to afford a colorless solid (4.8 g, 8.87 mmol); yield 99%. ^{1}H NMR ($CDCl_3$) δ 8.30 (s, br, NH), 7.40−7.27 (m, 10 H), 5.17 (s, 2H), 5.12 (s, 2H), 4. 64 (br s, NH), 3.62−3.40 (m, 5H), 2.16 (br s, 1H, NH), 1.70−1.55 (m, 6 H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 23. 2, 28.4, 28.8, 31.0, 40.7, 52.6, 65.7, 67.2, 68.2, 79.6, 127.9, 128.2, 128.4, 128.7, 128.8, 134.6, 136.7, 153.9, 156.0, 156.4, 163.7; LRMS: 543.13; HRMS: (ESI-MS *m*/*z*) M_{calc} for $C_{28}H_{39}N_4O_7$ 543.2819, found 543.2796.

Methyl *N*-(*o*,*p*-dinitrophenyl sulfonyl)-glycinate (4). To a stirred solution of glycine ester (13.1 g, 93.8 mmol) in dichloromethane (100 mL) was added 2,4-dinitrobenzenesulphonyl chloride (25 g, 93.7 mmol) at room temperature. Pyridine (15 mL, 187.6 mmol) was added to the reaction mixture over a 15-min span and stirred at ambient temperature for another 0.5 h. The organic layer was washed several times with water, followed by brine and dried over Na_2SO_4 (anhyd). The solvent was evaporated *in vacuo*, and the crude product was purified by column chromatography and dried to give a yellow solid (25 g, 75 mmol); yield 80%. ^{1}H NMR ($CDCl_3$) δ 8.74 (d, J = 2.3 Hz, 1H), 8.55 (dd, J_1 = 2.3 Hz, J_2 = 8.5 Hz, 1H), 8.29 (d, J = 8.5 Hz, 1H), 4.09−4.02 (m, 4H), 1.18 (t, J = 6.0 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 14.0, 45.0, 62.1, 121.0, 127.2, 132.2, 139.8, 147.8, 149.8, 168.7; LRMS: 332.00; HRMS: (ESI-MS) M_{calc} for $C_{10}H_{10}N_3O_8S$ 332.0189, found 332.0218.

Boc-Lys{(Guanidine-Di-Cbz)-Ψ[CH_2N(*o*,*p*-diNBS)]}Gly-OEt (5). To a stirred solution of *o*,*p*-DNBS-Gly-OEt **4** (1.55 g, 4.6 mmol), triphenyl phosphine (1.2 g, 4.6 mmol) and alcohol **3** (2.5 g, 4.6 mmol) in dry THF (20 mL) under inert atmosphere, cooled in an ice-bath, was added di-isopropylazidodicarboxylate (DIAD) (0.65 mL, 4.6 mmol) dropwise over a period of 0.5 h. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was evaporated *in vacuo*. The oily residue was purified by column chromatography and dried to afford a yellow solid (3 g, 3.5 mmol); yield 76%. ^{1}H NMR ($CDCl_3$) δ 8.45 (dd, J_1 = 2.4 Hz, J_2 = 8.8 Hz, 1H), 8.42 (d, J = 2.4 Hz, 1H), 8.40−8.23 (br s, NH), 8.22 (d, J = 8.5 Hz, 1H), 7.40−7.29 (m, 10H), 5.17 (s, 2H), 5.12 (s, 2H), 4.09−4.06 (m, 3H), 3.90−3.70 (m, 1H), 3.60- 3.30 (m, 4H), 1.58- 1.43 (m, 8 H), 1.41 (s, 9 H), 1.22 (t, J = 6.0 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 14.0, 23.0, 28.3, 28.7, 32.2, 40.6, 47.7, 47.8, 52.3, 61.7, 67.1, 68.2, 79.9, 119.5, 126.0, 127.9, 128.2, 128.3, 128.4, 128.6, 128.7, 128.7, 128.8, 129.8, 132.3, 134.6, 136.8, 149.6, 153.9, 156.0, 163.7, 168.5; LRMS: 856.80; HRMS: (ESI-MS *m*/*z*) M_{calc} for $C_{38}H_{47}N_7O_{14}S$, 857.2902, found 857.2984.

Boc-Lys{(Guanidine-Di-Cbz)[-Ψ(CH_2N)]}Gly-OEt (6). To a stirred solution of **5** (3.5 g, 4.08 mmol) in dichloromethane (20 mL) under inert atmosphere was added *n*-propyl amine (6.65 mL, 81.6 mmol). The resulting solution was stirred at room temperature

(47) Wimley, W. C.; White, S. H. *Biochemistry* **2000**, *39*, 4432–4442.
(48) Potocky, T. B.; Menon, A. K.; Gellman, S. H. *J. Am. Chem. Soc.* **2005**, *127*, 3686–3687.

for another 20 min. The solvent was evaporated. The crude product was purified by column chromatography and dried to afford a light yellow colored liquid (1.7 g, 3.1 mmol); yield 76%. ^{1}H NMR ($CDCl_3$) δ 8.28 (br s, NH), 7.38−7.25 (m, 10 H), 5.15 (s, 2H), 5.10 (s, 2H), 4.15 (q, J = 7.2 Hz, 2H), 3.70−3.50 (m, 1H), 3.50−3.25 (m, 4H), 2.60 (2ABq, J_1 = 12.2 Hz, J_2 = 10.4 Hz, 2H), 1.56−1.4 (m, 7H), 1.41 (s, 9H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 14.2, 23.2, 28.4, 28.8, 32.8, 40.8, 50.3, 50.9, 52.9, 60.7, 67.1, 68.0, 79.0, 127.8, 128.1, 128.3, 128.4, 128.6, 128.7, 134.6, 136.8, 153.8, 155.8, 156.0, 163.7, 172.4; LRMS: 628.13; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{32}H_{46}N_5O_8$ 628.3347, found 628.3323.

Boc-Lys(Guanidine-Di-Cbz)-PNA-Thyamine Monomer Ethyl Ester (7a). To a stirred solution of thymine acetic acid (0.48 g, 2.64 mmol) in dry DMF (15 mL) was added DCC (0.54 g, 2.64 mmol) and DhbtOH (0.42 g, 2.64 mmol). The resulting mixture was stirred at room temperature for another 1 h. Compound **6** (1.2 g, 2.2 mmol) dissolved in dry DMF (10 mL) was added to the reaction mixture and stirred at 40 °C for another 24 h. The solvent was removed under reduced pressure and the residue was diluted with ethyl acetate (100 mL). The ethyl acetate layer was washed with saturated solution of $NaHCO_3$ (100 mL), followed by 10% $KHSO_4$ (100 mL). The organic layer was washed with brine (50 mL) and dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The crude product was further purified by column chromatography and dried to afford a white solid (1.5 g, 1.88 mmol); yield 82%. ^{1}H NMR (DMSO d_6) δ 8.38 (br s, 1H), 7.44−7.23 (m, 10H), 7.21 (br s, 1H), 6.81−6.56 [2d (Rotamer$_{1,2}$),* J_1 = J_2 = 9.2 Hz, 1H], 5.19 (s, 2H), 5.01 (s, 2H), 4.80−4.40 [ABq (Rot$_1$, J = 16.2 Hz), m (Rot$_2$), 2H], 4.39−2.88 (m, 9H), 1.73 (s, 3H), 1.58−1.39 (m, 4H), 1.33 [d (Rot$_{1,2}$), J = 3.5 Hz, 9H], 1.15 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO d_6) δ 23. 2, 28.4, 28.8, 31.0, 40.7, 52.6, 65.7, 67.2, 68.2, 79.6, 127.9, 128.2, 128.4, 128.7, 128.8, 134.6, 136.7, 153.9, 156.0, 156.4, 163.7; LRMS: 794.04; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{39}H_{52}N_7O_{11}$ 794.3725, found 794.3682.

******Because of the carbonyl group in the bridge, two rotamers were formed for the monomers and the ester precursors—each with different chemical shift.* Adenine ester (**7b**), Guanine ester (**7c**), Cytosine ester (**7d**) were prepared and isolated following the same procedure as outlined in the preparation of (**7a**).

Boc-Lys(Guanidine-Di-Cbz)-PNA-Adenine (Cbz) Monomer Ethyl Ester (7b); Yield 85%. ^{1}H NMR (DMSO d_6) δ 8.60−8.56 [2d (Rot$_{1,2}$), J_1 = 2.1 Hz, J_2 = 1.6 Hz, 1H], 8.47−8.32 (m, 1H), 8.31−8.19 [2d (Rot$_{1,2}$), J_1 = 1.1, J_2 = 2.5 Hz, 1H], 7.65−7.25 (m, 15H), 6.95−6.56 [2d (Rot$_{1,2}$), J_1 = J_2 = 9.1 Hz, 1H], 5.42−4.90 (m, 8H), 4.60−3.70 (m, 6H), 3.56−3.37 (m, 2H), 3.07−2.91 (m, 1H), 1.59−1.41 (m, 4H), 1.35 [d (Rot$_{1,2}$), J = 2.8 Hz, 9H], 1.29−1.22 (m, 2H), 1.37 [2t (Rot$_{1,2}$), J_1 = J_2 = 7.04, 3H]; ^{13}C NMR (DMSO d_6) δ 14.4, 23.4, 28.6, 29.7, 31.5, 32.1, 44.3, 48.5, 49.2, 52.3, 60.8, 61.7, 65.6, 66.7, 66.8, 68.0, 78.4, 123.4, 128.2, 128.4, 128.7, 128.8, 129.0, 131.9, 132.0, 135.6, 136.8, 137.3, 145.4, 149.8; LRMS: 937.13; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{47}H_{57}N_{10}O_{11}$ 937.4208, found 937.4132.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Guanine (Cbz) Monomer Ethyl Ester (7c); Yield 80%. ^{1}H NMR (DMSO d_6) δ 8.42−8.31 (m, 1H), 7.69 [d (Rot$_{1,2}$), J = 6.5 Hz, 1H], 7.60−7.09 (m, 15 H), 6.89−6.58 [2d (Rot$_{1,2}$), J_1 = 9.5 Hz, J_2 = 8.3 Hz, 1H], 5.34−4.78 (m, 8H), 4.44−2.87 (m, 9H), 1.89−1.47 (m, 4H), 1.31 [d (Rot$_{1,2}$), J = 9.8 Hz, 9H], 1.22 (t, J = 7.0 Hz, 2H), 1.13 (t, J = 7.0 Hz, 3H); ^{13}C NMR (DMSO d_6) δ 14.4, 23.4, 24.9, 28.6, 28.7, 31.7, 33.8, 44.1, 48.0, 48.5, 49.3, 52.2, 60.9, 61.7, 66.8, 67.6, 68.0, 78.6, 119.5, 128.5, 128.8, 128.9, 129.0, 135.6, 136.0, 137.3, 140.5, 148.1, 150.1, 153.2, 155.5, 155.7, 156.1, 163.5, 167.2, 167.7, 169.3, 169.8; LRMS: 975.27; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{47}H_{56}N_{10}O_{12}Na$ 975.3977, found 975.3817.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Cytosine (Cbz) Monomer Ethyl Ester (7d); Yield 80%. ^{1}H NMR (DMSO d_6) δ 8.45−8.38 (m, 1H), 7.87−7.74 [2d (Rot$_{1,2}$), J_1 = J_2 = 7.5 Hz, 1H], 7.65−7.48 (m, 1H), 7.43−7.19 (m, 15 H), 7.06−6.91 [2d (Rot$_{1,2}$), J_1 = 9.2 Hz, J_2 = 8.0 Hz, 1H], 5.18 (s, 2H), 5.17 (s, 2H), 4.94−4.49 [2ABq (Rot$_{1,2}$), J_1 = J_2 = 16.3 Hz, 2H], 4.40−2.84 (m, 9H), 1.77−1.41 (m, 4H), 1.33 [d (Rot$_{1,2}$), J = 3.8 Hz, 9H], 1.21 (t, J = 7.1 Hz, 2H), 1.14 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO d_6) δ 14.4, 23.2, 23.4, 28.6, 31.5, 48.6, 49.3, 49.8, 52.2, 60.9, 61.6, 66.8, 66.9, 68.0, 78.4, 94.3, 128.3, 128.5, 128.7, 128.9, 129.0, 131.8, 132.0, 135.6, 136.4, 137.4, 150.9, 153.7, 155.3, 155.5, 156.0, 163.5, 167.8, 168.2, 169.2, 169.6; LRMS: 935.39; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{46}H_{56}N_8O_{12}Na$ 935.3915, found 935.3884.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Thyamine Monomer Acid (8a). To a stirred solution of **7a** (0.5 g, 6.3 mmol) in THF (10 mL), 2N NaOH (10 mL) was added dropwise at 0 °C over a period of 15 min. The resulting mixture was stirred at same temperature for another 0.5 h. At the end of the reaction, as monitored by TLC, H_2O (20 mL) was added. The reaction mixture was extracted with ethyl acetate (2 × 25 mL). The combined aqueous layers were acidified with 5% HCl (to pH 4) at 0 °C and then extracted with ethyl acetate (4 × 25 mL). The combined organic layers were dried over Na_2SO_4 (anhd.) and the solvent was evaporated *in vacuo*. The crude product was purified by column chromatography and dried to afford a colorless solid (0.35 g, 4.5 mmol); yield 72%. ^{1}H NMR (DMSO d_6) δ 8.42−8.39 (m, 1H), 7.43−7.23 (m, 10 H), 7.18 (br s, 1H), 6.80−6.60 [2d (Rot$_{1,2}$), J_1 = 8.2 Hz, J_2 = 7.4 Hz, 1H], 5.18 (s, 2H), 5.00 (s, 2H), 4.71−4.31 [ABq (Rot$_1$, J = 17.2 Hz), m (Rot 2), 2H], 3.94−2.60 (m, 7H), 1.7 (s, 3H), 1.55−1.36 (m, 3H), 1.32 (s, 9H), 1.29−1.10 (m, 3H); ^{13}C NMR (DMSO d_6) δ 12.4, 23.4, 28.7, 31.9, 48.1, 48.9, 49.2, 51.7, 52.2, 66.8, 68.0, 77.9, 78.2, 108.4, 128.2, 128.4, 128.7, 129.0, 135.6, 137.3, 142.6, 151.5, 153.1, 155.4, 156.0, 163.4, 164.8, 168.3; LRMS 766.27; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{37}H_{48}N_7O_{11}$ 766.3412, found 766.3429.

PNA monomer Adenine (Cbz) acid (**8b**), Guanine ester(Cbz) acid (**8c**) and Cytosine (Cbz) acid (**8d**) were prepared and isolated following the same procedure as outlined in the preparation of (**8a**) above.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Adenine (Cbz) Monomer Acid (8b); Yield 75%. ^{1}H NMR (DMSO d_6) δ 8.55 [d (Rot$_{1,2}$), J = 5.2 Hz, 1H], 8.42−8.37 (m, 1H), 8.23 [d (Rot$_{1,2}$) J = 11.8 Hz, 1H], 7.47−7.21 (m, 15 H), 6.96−6.61 [2d (Rot$_{1,2}$) J_1 = 9.5 Hz, J_2 = 7.9 Hz, 1H], 5.30−4.90 (m, 9H), 4.04−2.68 (m, 7H), 1.55−1.37 (m, 3H), 1.28 (s, 9H), 1.26−1.16 (m, 3H); ^{13}C NMR (DMSO d_6) δ 23.4, 28.7, 31.9, 44.5, 49.3, 51.9, 63.3, 66.7, 66.8, 68.0, 77.9, 123.3, 126.8, 127.0, 128.3, 128.4, 128.7, 128.8, 129.0, 135.6, 136.8, 137.4, 145.7, 149.7, 151.7, 152.6, 153.1, 155.4, 155.9, 163.4, 166.5, 167.6; LRMS: 909.00; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{45}H_{53}N_{10}O_{11}$ 909.3895, found 909.3897.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Guanine (Cbz) Monomer Acid (8c); Yield 68%. ^{1}H NMR (DMSO d_6) δ 8.42−8.39 (m, 1H), 7.72 [d (Rot$_{1,2}$) J = 5.2 Hz, 1H], 7.43−7.26 (m, 15H), 6.91−6.61 [2d (Rot$_{1,2}$), J_1 = 9.8 Hz, J_2 = 8.0 Hz, 1H], 5.22 (s, 2H), 5.15−4.78 (m, 4H), 4.19−2.85 (m, 7H), 1.58−1.37 (m, 3H), 1.31 [d (Rot$_{1,2}$), J = 6.1 Hz, 9H], 1.25−1.12 (m, 3H); ^{13}C NMR (DMSO d_6) δ 23.3, 23.4, 28.6, 28.7, 31.7, 44.2, 49.1, 66.8, 67.5, 67.6, 68.0, 78.0, 78.5, 119.5, 119.6, 128.2, 128.3, 128.6, 128.7, 128.8, 135.6, 135.9, 136.0, 137.3, 147.6, 147.7, 150.0, 153.2, 154.9, 155.0, 155.5, 155.6, 156.1, 163.4, 166.9, 167.6; LRMS: 925.13; HRMS: (ESI-MS *m/z*) M_{calc} for $C_{45}H_{53}N_{10}O_{12}$ 925.3845, found 925.3860.

Boc-Lys (Guanidine-Di-Cbz)-PNA-Cytosine (Cbz) Monomer Acid (8d); Yield 70%. ^{1}H NMR (DMSO d_6) 8.38 (br s, 1H), 7.80 (d, J = 7.5 Hz, 1H), 7.47−7.21 (m, 15 H), 6.97 (d, J = 7.3 Hz, 1H), 6.85−6.60 [2d (Rot$_{1,2}$), J_1 = 11.1 Hz, J_2 = 6.4 Hz, 1H], 5.17 (br s, 4H), 5.00 (s, 2H), 4.88−4.89 [ABq (Rot$_1$, J = 16.3 Hz), m (Rot$_2$), 2H], 3.98−2.68 (m, 7H), 1.56−1.36 (m, 3H), 1.32 (s, 9H), 1.29−1.10 (m, 3H); ^{13}C NMR (DMSO d_6) 23.4, 28.7, 31.9, 41.0, 49.0, 49.4, 50.1, 51.7, 52.3, 66.8, 66.9, 68.0, 77.9, 78.3, 94.4, 128.2, 128.3, 128.4, 128.6, 128.7, 128.9, 129.0,

135.6, 136.4, 137.3, 151.0, 151.4, 153.1, 153.6, 155.4, 155.9, 156.0, 163.4, 167.1, 168.1; LRMS: 885.40; HRMS: (ESI-MS *m*/*z*) M_{calc} for $C_{44}H_{53}N_8O_{12}$ 885.3783, found 885.3871.

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Supporting Information Available: General experimental details, salt-dependent T_ms of hybrid duplexes, and ^1H and ^{13}C spectra for compounds **2**−**8** and MALDI-TOF spectra of all the oligomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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