(α,α-dimethyl)glycyl (dmg) PNAs
Achiral PNA analogs that form stronger hybrids with cDNA relative to isosequential RNA

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The design and facile synthesis of sterically constrained new analogs of PNA having gem-dimethyl substitutions on glycine (dmg-PNA-T) is presented. The PNA oligomers [α-aminoisobutyric acid (aeg) and aminopropyl dimethylglycyl (apdmg)] synthesized from the monomers 6 and 12) effected remarkable stabilization of homothymine-PNA, homoadenine-DNA/RNA triples and mixed base sequence duplexes with target cDNA or RNA. They show a higher binding to DNA relative to that with isosequential RNA. This may be a structural consequence of the stericly rigid gem-dimethyl group, imposing a pre-organized conformation favorable for complex formation with cDNA.

The results complement our previous work that had demonstrated that cyclohexanyl-PNAs favor binding with cRNA compared with cDNA and imply that the biophysical and structural properties of PNAs can be directed by introduction of the right rigidity in PNA backbone devoid of chirality. This approach of tweaking selectivity in binding of PNA constructs by installing gem-dimethyl substitution in PNA backbone can be extended to further fine-tuning by similar substitution in the aminoethyl segment as well either individually or in conjunction with present substitution.

 Introduction
Peptide nucleic acid (PNA) is an interesting class of nucleic acid mimics first reported by Nielsen and Buchardt that is formally neither a peptide nor a nucleic acid, but embodies the hybrid structural features of both classes of bio-molecules. The structure of PNA consists of repeating units of 2-aminoethylglycine (aeg) to which the nucleobases (A, C, T and G) are attached via a tertiary amide linkage (Fig. 1). PNA hybridizes to cDNA and RNA sequences with almost equal avidity in a sequence specific manner. This feature, combined with its biological and chemical stability, promised potential applications for in vitro diagnostics and antisense therapeutics. PNA directed against genomic DNA around the transcription start site effectively knocks down the expression of the targeted gene and is an effective addition to the arsenal of gene silencing agents. Despite several inherent advantages like sequence-specific binding and resistance to cellular enzymes, the major limitations of PNA for its effective applications are poor solubility in aqueous media, inefficient cellular uptake, almost equal affinity to cDNA and RNA (reducing selectivity of target by half) and ambiguity in parallel/antiparallel orientation selectivity of binding (non-specific targeting). To overcome these limitations, several efforts have been made to structurally alter the aeg-PNA backbone. Previous efforts from this laboratory and that of others have resulted in a number of acyclic, cyclic and chiral backbone modifications to generate a variety of PNA analogs. The aeg-PNA backbone composed of C-C and C-N bonds is highly flexible with a capacity to reorganize slowly into the preferred conformation for hybridization with cDNA or RNA. Pre-organizing the aeg-PNA backbone into “hybridization-competent conformation” is expected to impart entropic advantages for selective or preferential binding to DNA or RNA. Indeed, rational modifications of aeg-PNA backbone by introduction of cis-1,2-disubstituted cyclohexyl moiety to match the dihedral angle of the lone C-C bond in ethylenediamine segment to 60° found in PNA:RNA duplexes, resulted in achieving significant discrimination in binding of complementary isosequential DNA and RNA with preference for binding to RNA. Similarly, the introduction of chirality into aeg-PNA backbone has shown more selectivity for parallel or antiparallel binding depending on the nature of modifications, while introduction of cationic side-chains have improved solubility and enhanced cell uptake. A simpler way to impart steric constrain without incorporating rigid and chiral cyclic moieties is to introduce gem-dimethyl substitution into the flexible aminopropylglycine backbone of PNA (Fig. 2A and B). Such acyclic, achiral backbone having gem-dimethyl...
function would not only be rigid, but if the steric constraints imposed lead to favorable pre-organization, the inherent binding to cDNA or RNA will be enhanced due to entropic factors. The gem-dimethyl substitution on glycine segment of PNA can be achieved by replacing the glycine in backbone with α-aminoisobutyric (aib) moiety to obtain the aminopropyl-(α,α-dimethyl)glycyl (aedmg)-PNA (Fig. 2A). A number of aib-containing peptides occur naturally and the gem-dimethyl substitution on α-carbon is well known to promote helices in polypeptides. This feature, although not directly extendible to PNA (which is not a classical peptide), provided an initial rationale for us to synthesize the gem-dimethyl substituted aedmg-PNA. To examine the correlated effects of steric constraints developed due to gem-dimethyl(glycine) on the backbone in the adjoining aminopropyl segment in backbone, PNA analogs having aminopropyl moiety (Fig. 2B, R = Me, aminopropyl-(α,α-dimethyl)glycyl (apdmg)-PNA) were also synthesized for comparative studies of the DNA/RNA complementation abilities. The unsubstituted aminopropylglycine-PNA (Fig. 2C, αg-PNA) was reported to induce destabilization of the derived PNA:DNA hybrid. Synthesis of PNA with spirocyclopentyl substitution (Fig. 2D) on glycine is known in literature, but no data on its incorporation developed due to gem-dimethyl glycine on the backbone is well known to promote helices in polypeptides. It was demonstrated here that the incorporation of α,α-dimethyl substitution on glycine in PNA stabilizes the derived PNA:DNA and PNA:RNA hybrids with significant preference for the PNA:DNA binding.

Results and Discussion

Synthesis of dmg-PNA-T monomers (6 and 12). A straightforward route to obtain the desired dmg-PNA monomers 6 and 12 would be to directly N-alkylate the easily accessible ethyl α-aminoisobutyrate (H-N-CH₂-COOEt, aib). However, N-alkylation of aib with Boc-HNCH₂CH₂Cl or by reductive alkylation of Schiff base obtained by condensation with BocHNCH₂CHO was not successful (Supplemental Material). Since this may be due to the steric problems imposed by the gem-dimethyl group, it was thought to reverse the N-alkylation strategy by using the gem-dimethyl component as the alkylating agent to react with N-protected diethylamine (Fig. 3A). The synthesis of aedmg-PNA-T target monomer 6 was performed starting from 1,2-diaminomethane 1 which was treated with Boc-anhydride to give the N1-Boc-1,2-diaminoethane 2 that was alkylated at N2 with ethyl-2-bromoisobutyrate to obtain aminoethyl-(α,α-dimethylglycine) ester 3 in moderate yields. This was reacted with chloroacetyl chloride to yield the corresponding N-chloroacetyl chloride derivative 4 which was used for alkylation to obtain N-(Boc-aminoethyl-α,α-dimethylglycyl)-chymine (αpdmg) ester 5. It was hydrolyzed by methanolic sodium hydroxide to yield the desired aedmg-PNA monomer acid 6 for use in PNA synthesis (Fig. 3).

The synthesis of the homologous aminopropyl (α,α-dimethyl)glycine (apdmg) acid monomer 7 (Fig. 3B) was performed starting from 1,2-diaminopropane by following the same route as in Figure 3A, through the intermediate steps yielding the products 8–10. The structural integrity of all new compounds of Figure 3 was confirmed by 1H, 13C NMR spectroscopic analysis and mass spectrometry (Supplemental Material).

Solid phase synthesis of aedmg-PNA and apdmg-PNA oligomers. PNA oligomers containing gem-dimethylglycyl unit (dmg-PNA-T) were assembled by solid-phase peptide synthesis on MBHA resin derivatized with lysine and the sequences are shown in Table 1. The unmodified PNA (apo-PNAs 1 and 12) and the modified dmg-PNA-T oligomers (Table 1, aedmg-PNA 2–6 and 13 and apdmg-PNA 7–11 and 14) were designed to test the effect of dmg modifications on the stability of the derived PNA:DNA and PNA:RNA duplexes and triplexes. The aedmg-PNA-T unit (t) was incorporated into the apo-PNA-T₆ sequence at pre-defined positions by solid phase synthesis to yield aedmg-PNAs (PNA 2–5) and the all-modified aedmg-PNA 6. Similarly, the apdmg-PNA-T unit (t) was incorporated to obtain the apdmg-PNA-T oligomers (PNA 7–11). The mixed base PNA sequences having three modified units of apdmg-PNA-T (t) (PNA 13) and aedmg-PNA-T (t) (PNA 14) were synthesized along with the unmodified apo-PNA 12 to examine the effect of modifications on the stability of PNA:DNA and PNA:RNA duplexes. All PNA oligomers were cleaved from the solid support by treatment with TFA-TFMSA to yield PNA carrying lysine at the carboxyl termini (to enhance their aqueous solubility). The PNA oligomers were purified by HPLC on a PepRPC column and finally characterized by MALDI-TOF mass spectrometry (Table 1). The complementary homoadeninyl DNA 15 having a CG/GC lock at the ends to prevent slippage in triplexes and the
mixed base sequences (Table 1, footnote) DNA 16–18 complementary to PNAs 13 and 14 were synthesized on a DNA synthesizer using standard phosphoramidite chemistry. Poly rA (RNA 19) was used for triplex studies with PNA oligomers 1–11. The mixed base RNA oligonucleotides RNA 20 and 21 (Table 1, footnote) complementary to PNA oligomers (13 and 14) were obtained from commercial sources along with their HPLC purity and mass spectral data.

Figure 3. Synthesis of dmg-PNA monomers (A) aedmg-PNA-T monomer 1 and (B) apdmg-PNA-T monomer 12. Values in brackets indicate yields. In Scheme B reagents a–e are same as in Scheme A.

Table 1. PNA sequences with their HPLC and mass spectral data and cDNA and RNA sequences*

| Entry | PNA sequence | HPLC Rf | Mol Formula | Mol. Wt. Calc | Mol. Wt. Obs |
|-------|--------------|---------|-------------|---------------|--------------|
| 1.     | PNA 1 H-H-TTTTTTT-LysNH₂ | 7.34    | C₉₄H₁₂₇N₃₅O₃₃ | 2275.25      | 2275.47      |
| 2.     | PNA 2 H-TTTTTT-LysNH₂ | 8.02    | C₉₆H₁₃₁N₃₅O₃₃ | 2303.30      | 2303.43      |
| 3.     | PNA 3 H-TTTTTT-LysNH₂ | 8.32    | C₉₈H₁₃₅N₃₅O₃₃ | 2331.36      | 2334.60      |
| 4.     | PNA 4 H-TTTTYTTT-LysNH₂ | 8.96    | C₁₀₀H₁₄₀N₃₅O₃₃ | 2370.40      | 2389.62      |
| 5.     | PNA 5 H-TTTYTTT-LysNH₂ | 9.30    | C₁₀₂H₁₄₇N₃₅O₃₃ | 2408.47      | 2436.78      |
| 6.     | PNA 6 H-TTTTTTT-LysNH₂ | 10.32   | C₁₁₀H₁₅₉N₃₅O₃₃ | 2509.68      | 2505.00      |
| 7.     | PNA 7 H-TTTTTTT-LysNH₂ | 8.63    | C₉₇H₁₃₃N₃₅O₃₃ | 2317.33      | 2316.78      |
| 8.     | PNA 8 H-TTTTTTT-LysNH₂ | 8.78    | C₁₀₀H₁₄₈N₆₀O₃₁ | 2559.41      | 2563.74      |
| 9.     | PNA 9 H-TTTTTTT-LysNH₂ | 9.65    | C₁₀₂H₁₵₁N₆₀O₃₁ | 2401.49      | 2452.61      |
| 10.    | PNA 10 H-TTTTTTT-LysNH₂ | 10.16   | C₁₀₅H₁₵₄N₆₀O₃₁ | 2444.57      | 2444.60      |
| 11.    | PNA 11 H-TTTTTTT-LysNH₂ | 10.00   | C₁₀₇H₁₵₇N₆₀O₃₁ | 2481.69      | 2481.21      |
| 12.    | PNA 12 H-TTTTTTT-LysNH₂ | 10.39   | C₁₀₉H₁₶₀N₆₀O₃₁ | 2528.81      | 2526.93      |
| 13.    | PNA 13 H-G-GAGACG-LysNH₂ | 13.50   | C₁₀₂H₁₵₀N₆₀O₃₁ | 2938.97      | 2941.47      |
| 14.    | PNA 14 H-G-GAGACG-LysNH₂ | 14.42   | C₁₀₄H₁₶₀N₆₀O₃₁ | 2981.05      | 2978.59      |

*T aeg, t aedmg and t apdmg PNA. DNA 15 CGAAAAAAGCC; DNA 16 CGAAAAAGAAGC; DNA 17 = AGTGTACT and DNA 18 = CATCTAGTA; RNA 19 poly rA, RNA 20 = AGUGAUCUC and RNA 21 = CAUCUAGUGA. In most PNA oligomers observed mass correspond to either M+nH⁺ (n = 1–5) or M + Na⁺.
cDNA-dA₈ are known to form PNA₂-DNA triplexes. The binding stoichiometry of apdmg-PNA₆-DNA₁₅ complexes was established as 2:1 expected for a PNA₂-DNA triplex from UV Job’s plot (see Supplemental Material). The thermal stabilities of triplexes from unmodified PNA₁ and apdmg-PNA₆ (PNA 2–11) with cDNA₁₅ and RNA₁₉ (polyribosomal) were determined from temperature-dependent UV absorbance at 260 nm. The UV-Tₘ plots show a single sigmoidal transition, characteristic of PNA₂-DNA triplex melting, wherein both the PNA strands dissociate simultaneously from DNA in a single step. The derivative plot of selected UV-melting data are shown in Figure 4 and the Tₘ values corresponding to peaks from such plots for various PNA₂-DNA and PNA₂-RNA triplexes of apdmg and apdmg-PNAs with cDNA₅ or RNA₁₉ are shown in Table 2.

It is seen from the UV-melting data (column 1) that introduction of single apdmg-PNA₆-T modification at N-terminus enhanced the Tₘ of derived PNA₂-DNA₁₅ triplexes by 8.1°C (entry 2) over unmodified triplex (entry 1) and a successive increase in the number of modifications (entries 3–6) effected stepwise enhancement in stability. Since each triplex has two PNA strands, the total number of modifications per triplex is twice that number in a single PNA strand and this corresponds to an average stabilization of around 3.5–4.5°C per modification in triplexes. Significantly, the fully modified oligomer (apdmg-PNA₆-T₈) formed triplex with DNA₁₅ (entry 6) so strongly that no melting was seen even up to 85°C. A progressive increase in Tₘ per unit of apdmg-PNA₆-T modification in homothymine sequences suggests some of the structural factors causing stabilization is additive and cumulative.

Since PNA₂-RNA triplexes have slightly different conformational features, the stabilizing effect of the apdmg-PNA units on the derived triplexes (PNA₂-RNA₁₉) was examined (Table 1, column 2). Single N-terminal modification gave only marginal positive effect (ΔTₘ = 1.4°C, entry 2) over control triplex (entry 1), while further increase in the number of apdmg-PNA units (entry 3–6), gave a Tₘ enhancement of about 2–2.5°C per modification. Again, PNA₆₉-RNA₁₉ triplex from the fully modified apdmg-PNA (entry 6) failed to show complete melting even at 85°C; it was noticed that the melting of apdmg-PNA-DNA triplexes with PNAs 6 and 5 having three or more modifications had a slightly larger change in hyperchromicity compared with unmodified PNA₂-DNA triplex suggesting that apdmg-modified PNAs have better stacking of the bases. It should be pointed out that the Tₘs for DNA and RNA triplexes are not directly comparable since RNA used is poly rA. Nevertheless, the relative trends are similar and the enhancement per modification in RNA triplexes is less than that for DNA triplexes.

To examine whether the observed enhancement in stability is at the cost of selectivity, the triplex Tₘ of completely modified apdmg-PNA₆-T₈ (PNA₁₉) with DNA₁₆ having single mismatch in the middle (C instead of A) was measured. A larger destabilization (ΔTₘ = -24.4°C, entry 8) was observed for mismatched triplex from fully modified apdmg-PNA triplex (entry 6) compared with triplex from unmodified control PNA₇ (ΔTₘ = -12.8°C, entry 7). This not only indicated the formation of triplexes by fully modified PNA₆ (although no melting was seen till 85°C) but also confirmed that the stability of apdmg-PNA-DNA triplex was not achieved at the expense of loss of sequence selectivity.

The UV-Tₘ data in Table 2, columns 3 and 4 are for the corresponding triplexes derived from apdmg-PNA₆-T₈ oligomers, having propylene chain instead of ethylene in the apdmg-PNA oligomers (PNA 7–11). While single N-terminal modification resulted in a slight destabilization, further modifications enhanced the Tₘ by 1.3°C per modification, which is less than 7–9°C seen for triplexes from apdmg-PNA (PNA 2–6). PNA₂-RNA triplexes derived from apdmg-PNA showed destabilization up to two modifications, but increasing them stabilized the triplexes over control. However, the degree of stabilization with apdmg-PNAs was lower compared with that seen with apdmg-PNA-RNA triplexes. The stabilization of apdmg-PNA-DNA/RNA triplexes is interesting, since it was earlier reported that aminopropylglycyl-PNA destabilized the derived hybrids compared with aminomethylglycyl-PNA. Thus, the gem-dimethyl substitution on glycine in both aminomethyl and aminopropyl PNAs stabilize the
The PNA:DNA and PNA:RNA duplexes differ in conformation and structural features compared to unmodified PNA and the stabilization is significantly better for constituted DNA and RNA triplexes over that of triplexes of unmodified PNA (see Table 1 for sequence). This is not possible in triplexes wherein the identity of parallel-antiparallel binding orientation of the two PNA strands involved in complex formation cannot be distinguished.

The dupplexes were constituted by mixing equimolar amounts of aedmg-PNA12, aedmg-PNA13 and apdmg-PNA14, individually with the cDNA17, RNA20 for antiparallel dupplexes and DNA18, RNA21 for parallel PNA:DNA dupplexes. The Job's plot completely supported formation of 1:1 dupplexes in both aedmg-PNA13 and apdmg-PNA14 complexes with DNA17 (see Supplemental Material). The Tm data (Table 3) was obtained from the plot of UV absorbance vs temperature and show that the antiparallel dupplex (aedmg-PNA13:DNA17) with 3 modifications exhibits very high stabilization (entry 1, DTm = +31.1°C, 10°C/modification) compared with the corresponding unmodified dupplex (DNA12:DNA17, entry 1). The parallel dupplex of aedmg-PNA13 (DNA13:DNA18) is also significantly modified (entry 2, DTm = +19°C, 6°C/modification) compared with the unmodified parallel dupplex (DNA12:DNA18, entry 2). apdmg-PNA14 showed enhanced stabilization of its antiparallel dupplex with cDNA17 (entry 1, DTm = +25°C, 7°C/modification) over that of unmodified PNA12, but about the same stability for parallel dupplexes (entry 2).

The mixed unmodified PNA12 and the modified aedmg- apdmg-PNA13 also forms antiparallel and parallel dupplexes with

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**Table 2.** UV-Tm (°C) of aedmg-PNA2:DNA and apdmg-PNA2:RNA triplexes*

| Entry | PNA sequence | No | 1 = aedmg | 2 = apdmg |
|-------|--------------|----|-----------|-----------|
|       | No | DNA15 | RNA19 | DNA15 | RNA19 |
| 1     | H-TTTTTTTT-LysNH₂ | 1 | 43.0 | 53.8 | 43.0 | 53.8 |
| 2     | H-TTTTTTTLys NH₂ | 2 | 51.1 (+18.1) | 55.2 (+14.0) | 7 | 40.3 (-2.7) | 44.3 (+9.5) |
| 3     | H-TTGTGTGT-Lys NH₂ | 3 | 57.9 (+14.9) | 64.7 (+10.9) | 8 | 48.4 (+5.4) | 51.1 (-2.7) |
| 4     | H-TTGTGTGT-Lys NH₂ | 4 | 64.7 (+21.7) | 67.4 (+13.0) | 9 | 56.2 (-12.2) | 55.2 (+1.4) |
| 5     | H-TTGTGTGT-Lys NH₂ | 5 | 79.0 (+36.6) | 72.8 (+19.0) | 10 | 59.2 (+16.2) | 56.5 (+2.7) |
| 6     | H-TTTTTTTTT-Tys NH₂ | 6 | > 85 (> 43) | > 85 (> 32) | 11 | 76.9 (+33.9) | 60.6 (+41) |
| 7     | H-TTTTTTTTT-LysNH₂ | 7 | 30.3 (-12.0) | 61.6 (-24.6) | 8 | 30.2 (-12.0) | 61.6 (-24.6) |

*Experiments were repeated at least thrice and the Tm values are accurate to (±) 0.5°C. T and t indicate aedmg and apdmg-PNA respectively. Values in brackets denote degree (±) of stabilization (+) or destabilization (-) compared with control PNA1. Numbers in bold represent PNA (see Table 1 for sequence). DTm refers to complex with mismatch DNA16.

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**Table 3.** UV-Tm (°C) of DNA/RNA and aedmg/PNA:DNA/RNA dupplexes

| Entry | DNA/RNA | No | DNA17 | RNA12 | DNA18 | RNA13 | DNA19 | RNA14 |
|-------|---------|----|-------|-------|-------|-------|-------|-------|
| 1     | DNA17   | Tm | 49.8  | 80.9 (+31.1) | 72.8 (+23.0) |
| 2     | DNA18   | 37.5 | 56.5 (+19.0) | 49.7 (+12.2) |
| 3     | DNA19   | 50.1 | 72.3 (+12.2) | 64.7 (+14.6) |

*Tm values are average of at least three experiments and are accurate to (±) 0.5°C. Values in parenthesis indicate amount in degrees of stabilization (+) or destabilization (-) over unmodified PNA. Numbers in brackets denote antiparallel and parallel orientation respectively DNA/RNA sequences from right to left denote 5'-3' while PNA sequences correspond to N-C-terminus.
the complementary RNAs 20 and 21 respectively. The T_m data of these duplexes are shown in Table 3 (entry 3 and 4). While both aedmg and apdmg PNAs stabilize the corresponding anti-parallel PNA:RNA duplexes (entry 3, aedmg ΔT_m = +22.2°C and apdmg ΔT_m = +14.6°C) they actually destabilized the corresponding parallel PNA:RNA duplexes (entry 4) by 8.9°C and 7.6°C respectively compared with the unmodified PNA. Thus the T_m results clearly suggest that both aedmg and apdmg-PNAs enormously stabilize (1) duplexes with DNA and RNA over that of unmodified PNA, (2) antiparallel duplexes with DNA and RNA are more stable than the corresponding parallel duplexes and (3) show stronger preference for duplexing with cDNA relative to isosequential RNA.

It was generally observed that the width of the derivative UV/T_m curves of the complexes increased with the degree of substitution, which suggests slow association-dissociation equilibrium due to dmg substitution. However, the change in the intensity of the different derivative curves could not be correlated with the number of modifications in triplexes and perhaps a consequence of differential changes in base stacking for each modification. It is possible that all modified (PNA 6 and 12), and alternative (PNA 5 and 11) site-modified triplexes induce base stacking patterns in phase toward a global uniformity, while changes from single or random modifications may result in only local effects. However, in cases of duplexes, all modified aedmg-PNA 13 and apdmg-PNA 14 showed much higher amplitudes of first derivative curves indicating much better stacking in the dmg-PNA:DNA duplexes.

CD studies on apdmg-PNA:DNA and apdmg-PNA:RNA complexes. In order to examine if aedmg-PNAs have any significant differences in their overall conformational features, the CD spectra of PNA complexes with DNA were examined (Fig. 6). The PNA2:DNA triplexes exhibit two maxima at 260 nm and 280 nm, a minimum at 246 nm and crossover points around 250 nm. The double hump profile in the region 260–280 nm is characteristic of polyT-polyA:polyT (PNA 2:DNA) triplexes.28 The CD profiles of dmg-PNA:DNA duplexes were similar to those of the control achiral aeg-PNA:DNA duplexes indicating that the overall conformational effects induced by dmg-PNA-T units in the derived PNA complexes with cDNA seem to be only marginal.

The aib residues in peptides severely restrict the possible rotations about N-Cα and Cα-C' bonds toward promoting α-helix or 3 10 helix and the non-chiral nature of substitution may induce right or left handed structures equally well, depending on the nature of other residues in sequence.29 aedmg-PNA is not a classical peptide and hence the exact molecular origin and details of structural consequences of gem-dimethyl substitution in PNA backbone remains to be seen. The comparative studies on aedmg and apdmg-PNA duplexes clearly suggest that applying conformational constrain on glycine segment by introduction of gem-dimethyl substitution leads to both better hybridization by pre-organizing the backbone for better stacking, interaction of the base pairs. This gem-dimethyl effect on glycine is transmitted to adjacent ethylenediamine/propylenediamine chain since unlike ap-PNA (with only adjacent glycine in backbone), which destabilize the DNA complexes, apdmg-PNA stabilize both DNA and RNA hybrids.

Materials and Methods

For general experimental conditions see Supplemental Material. Mono-N-Boc-diaminoethane (2). 1,2-diaminoethane (1) (20 g, 0.33 mol) was taken in dioxane:water (1:1, 500 ml) and cooled in an ice bath. Boc-azide (5 g, 35 mmol) in dioxane (50 ml) was slowly added with stirring and the pH was maintained at 10.0 by continuous addition of 4M NaOH. The mixture was stirred for 8 h and the resulting solution was concentrated to 100 ml. The N1, N2- di-Boc derivative not being soluble in water, precipitated, and it was removed by filtration. The corresponding N1-mono-Boc derivative was obtained by repeated extraction from the filtrate in ethyl acetate. Removal of solvents yielded the mono-N-Boc-diaminoethane 2.
from which the former compound was precipitated and removed. Chloroacetyl chloride (3.5 ml, 43.8 mmol) was taken in 10% aqueous Na2CO3 (75 ml) in the presence of anhydrous K2CO3 (5.5 g, 40 mmol) and the mixture was stirred at 70°C for 15 h. The reaction mixture was concentrated to remove the acetic acid and work-up with ethylacetate-water and purification by silica gel column chromatography gave ethyl-N-Boc-(aminomethyl)-α,α-dimethylglycinate (3) as a yellowish oil (4.2 g, 76.6% yield). 1H NMR (CDCl3): δ: 5.29 (br s, 1H, N–), 4.95 (br s, 1H, N–), 2.13–3.12 (t, 2H), 2.51–2.95 (t, 2H), 1.63–1.56 (2t, 1H), 1.40 [s, 9H, C(CH3)2], 1.26 [s, 6H, C(CH3)2], 1.23–2.10 (t, 3H). 13C NMR (CDCl3): δ: 176.8, 159.9, 66.7, 58.8, 41.9, 38.5, 36.5, 28.5, 25.2, and 14.2 M+ (M+H).

Ethyl N-Boc-(aminomethyl)-α,α-dimethylglycinate (9). The reaction was complete within an hour and was brought to pH 8.0 by addition of 10% aqueous Na2CO3 and concentrated to remove the dioxane. The product after work-up was purified by column chromatography to obtain the ethyl N-Boc-(aminomethyl)-N-(chloroacetyl)-α,α-dimethylglycinate (4) as a yellowish oil (4.2 g, 82% yield). 1H NMR (CDCl3): δ: 5.13 (br s, 1H, N–), 4.27–4.17 (q 2H), 4.13 (t, 2H), 3.57–3.5 (t 2H), 3.36–3.30 (t 2H), 1.44–1.31 (t, 3H), 1.08 (s, 9H, C(CH3)2). 12.7–1.20 (t, 3H). 13C NMR (CDCl3): δ: 173.9, 167.3, 156.0, 79.8, 72.6, 61.6, 61.2, 43.7, 41.8, 41.2, 28.2, 23.8 and 13.9 M+ (M+H).

Ethyl N-Boc-(aminomethyl)-N-(chloroacetyl)-α,α-dimethylglycinate (15) (3.6 g, 12.5 mmol) reacted with chlorosacchar chloride (5 ml, 62.5 mmol) under similar conditions, work up and chromatographic purification gave ethyl N-Boc-(aminopropyl)-N-(chloroacetyl)-α,α-dimethylglycinate 16 as a yellowish oil (3.8 g, 83.5% yield). 1H NMR (CDCl3): δ: 4.77 (br s, 1H, N–), 4.17–4.06 (q 2H), 4.04 (s, 2H), 3.48–3.40 (t, 2H), 1.13–2.1 (t, 2H), 1.91–1.92 (s, 2H), 1.46 (s, 6H, C(CH3)2). 1.83 [s, 9H, C(CH3)2], 1.23–1.17 (t, 3H), 1.173.6, 164.2, 151.2, 110.6, 61.9, 61.1, 47.2, 41.9, 41.0, 32.2, 28.3, 23.7, and 13.8 M+ (M+H).

Ethyl N-Boc-(aminomethyl)-N-(chloroacetyl)-α,α-dimethylglycinate 4 (0.9 g, 11.4 mmol) was stirred with anhydrous K2CO3 (1.68 g, 12.2 mmol) and thymine (1.58 g, 12.5 mmol) in DMF. After completion of reaction (TLC), solvent DMF was removed under reduced pressure followed by aqueous work-up and column chromatographic purification gave N-Boc-(aminomethyl)-α,α-dimethyl glycerol-thymine ethyl ester 5 (4.8 g, 96%) as white solid (2.2 g, 15%). 1H NMR (CDCl3): δ: 3.98 (br s, 1H, N–), 2.65–2.63 (t, 2H), 1.57 [s, 9H, C(CH3)2]. 1.23–2.10 (t, 3H). 13C NMR (CDCl3): δ: 174.7, 166.4, 164.2, 151.2, 110.6, 61.9, 61.1, 47.2, 41.9, 41.0, 32.2, 28.3, 23.7, 14.2 (M+H).

Hydrolysis of the PNA ethyl esters 5 and 11. The ethyl ester 5 was hydrolyzed using aqueous NaOH (2N, 5 ml) in methanol (5 ml) and the resulting acid was neutralized with activated Dowex-1® till the pH of the solution was 7.0. The resin was removed by filtration and the filtrate was concentrated to obtain the resulting Boc-protected acid 6 in excellent yield (85%). 1H NMR (CDCl3): δ: 10.1–11.2H, thymine NH (as COOH), 7.18 (s, 1H), 5.43 (br s, 1H, NH2), 4.2–4.16 (q 2H), 3.5–3.16 (s, 2H), 3.39–3.32 (t, 2H), 1.87 (s, 3H, CH3), 1.48 [s, 6H, C(CH3)2], 1.23–1.16 (t, 3H). 13C NMR (CDCl3): δ: 173.7, 166.4, 164.2, 151.2, 110.6, 61.9, 61.1, 41.9, 31.9, 28.3, 23.8, 14.3, 12.3 M+ (M+H).

Solid phase synthesis. The solid phase synthesis was performed on MBHA resin (Sigma, 100–200 mesh) having initial amine loading value of 0.85 mmol/g, which was lowered to 0.25 mmol/g by capping with acetic anhydride in dry DME/DCM and pyridine as a base. The MBHA resin with free amine was functionalized by coupling with N-(β-Boc)-N(i)-Cys-1-lysine using DCCl as coupling reagent. The lysine loaded resin was swelled in dry...
CH₂Cl₂ (30 min) and treated with 50% TFA in CH₂Cl₂ (1 mL × 2, 15 min each). After washing with CH₂Cl₂, neutralization of TFA salt with 5% DIEPA in CH₂Cl₂ (1 mL × 3, 2 min each) and washed further with CH₂Cl₂. The unmodified/modified PNA oligomers were coupled to resin in presence of HBTU/HOBt/DIEPA in DMF/NMP and the synthesis continued using the same coupling agent to make PNA oligomers (Table 1). The efficiency of each coupling was monitored by Kaiser’s test. At the end of the assembly the PNA oligomers were cleaved from the resin with TFA/TMSA in presence of an excess agent thioanisole. A typical cleavage reaction consisted of treating of resin-bound PNA oligomer (10 mg) in an ice bath with thioanisole (20 µl) and 1,2-ethanedithiol (8 µl) for 10 min and TFA (120 µl) followed by TMSA (16 µl) and stirring continued for 2 h. After filtering the resin, the filtrate was evaporated under vacuum. The residue was dissolved in methanol (~0.1 mL) and precipitated by adding ether. The PNA oligomers were purified by RP-HPLC (C18 column) and characterized by MALDI-TOF mass spectrometry. The overall yields of the crude PNA oligomers were in the range 70–80%.

UV-Vis Measurements. The concentration of PNA and DNA was calculated on the basis of absorption using the molar extinction coefficients of nucleotides for A = 13.4, 1 = 9.8, C – 7.3 and G = 17.1 M cm⁻¹. The complexes were prepared in sodium phosphate buffer (10 mM, pH 7.4) by annealing the samples at 85°C for 5 min followed by slow cooling to room temperature. Absorbance vs. temperature profiles were obtained by monitoring at 260 nm with a UV-Vis spectrophotometer. Scans were taken at 5 to 85°C at a jump rate of 0.2°C per minute. Experiments were repeated at least thrice and the Tm values were obtained from the peaks in the first derivative plots. The reported Tm values are accurate to ±0.5°C.

Conclusions

In summary, the incorporation of gem-dimethyl substituted aminog-PNA monomer into the aminog-PNA backbone preferentially increased the Tm of the derived complexes with DNA as compared with RNA. This perhaps arises from a structural preorganization of the backbone due to steric constraints imposed by the gem-dimethyl unit leading to a better base stacking of base pairs in DNA/RNA complexes. This is interesting since the earlier study from our group has shown that the cyclic cyclohexanyl PNAAs show strong preference for RNA binding and the present results allow tuning of PNA backbone to DNA binding by inhibition of RNA without any associated chirality. Further work is in progress in our group to extend these features toward the study of PNA derivatives with the gem-dimethyl substitution shifted to the adjacent amino-aryl segment, combining substitution in both fragments, thermodynamic analysis and molecular dynamic simulations of the derived PNA-DNA and PNA-RNA duplexes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found at the following:
www.landesbioscience.com/journals/artificialdna/article/19185

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