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(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 [aminoethyl dimethylglycyl (*aedmg*) and aminopropyl dimethylglycyl (*apdmg*)] synthesized from the monomers 6 and 12) effected remarkable stabilization of homothyminePNA_2:homoadenine DNA/RNA triplexes 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 sterically 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[1] 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.[2,3] This feature, combined with its biological and chemical stability, promised potential applications for in vitro diagnostics and antisense therapeutics.[4-7] 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.[8,9]

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).[10] To overcome these limitations, several efforts have been made to structurally alter the *aeg*-PNA backbone. Previous efforts from this laboratory[11-14] and that of others[15,16] 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.[17-19] Similarly, the introduction of chirality into *aeg*-PNA backbone has shown more selectivity for parallel or antiparallel binding depending on the nature of modifications,[11,12] while introduction of cationic side-chains have improved solubility and enhanced cell uptake.[20-22]

A simpler way to impart steric constrain without incorporating rigid and chiral cyclic moieties is to introduce *gem*-dialkyl substitution into the flexible aminoethylglycine backbone of PNA (**Fig. 2A and B**). Such acyclic, achiral backbone having *gem*-dialkyl

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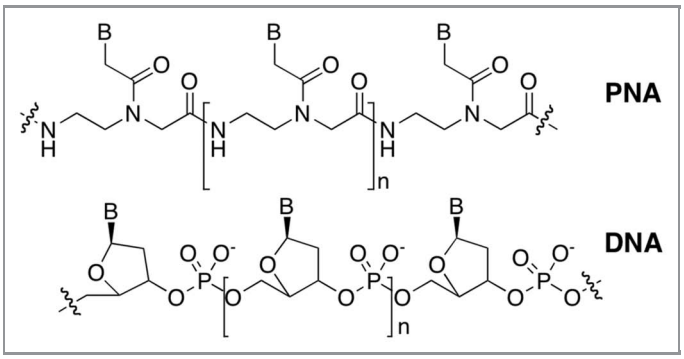

Figure 1. General structures of PNA and DNA: B, nucleobase (T/A/C/G).

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 aminoethyl-*(*α,α-dimethyl)glycyl (*aedmg*) PNAs (**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.[23] 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 constrain developed due to gem-dimethyl glycine on the backbone in the adjoining aminoethyl 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**, *apg*-PNA) was reported to induce destabilization of the derived PNA:DNA hybrids.[24] Synthesis of PNA with spirocyclopentyl substitution (**Fig. 2D**) on glycine is known in literature,[25,26] but no data on its complementation properties are reported. It is 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_2N-CMe_2-COOEt, *aib*). However, N-alkylation of *aib* with Boc-$HNCH_2CH_2Cl$ or by reductive alkylation of Schiff base obtained by condensation with BocHN-CH_2-CHO 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 dialkylamine (**Fig. 3A**). The synthesis of *aedmg*-PNA-T target monomer **6** was performed starting from 1,2-diaminoethane **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-dimethyl)glycine ethyl ester **3** in moderate yields. This was reacted with chloroacetyl chloride to yield the corresponding N-(chloroacetyl) derivative **4** which was used for alkylation to obtain *N*-(Boc-aminoethyl-α,α-dimethylglycyl)-thymine ethyl 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, ^{13}C NMR spectroscopic analysis and mass spectrometry (**Supplemental Materials**).

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 (*aeg*-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 *aeg*-PNA-T_8 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 *aedmg*-PNA-T (**t**) (PNA **13**) and *apdmg*-PNA-T (***t***) (PNA **14**) were synthesized along with the unmodified *aeg*-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 PNAs carrying lysine at the carboxyl terminus (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

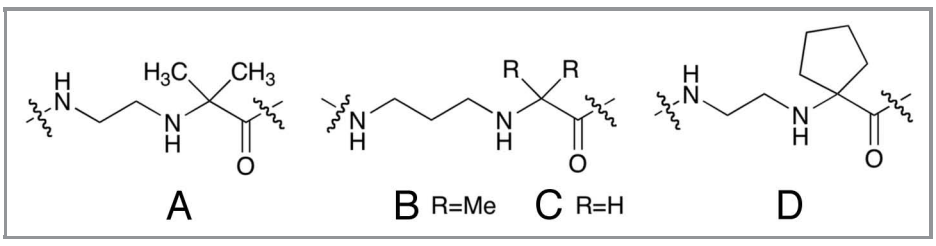

Figure 2. Structures of modified PNAs (A) *aedmg*, (B) *apdmg*, (C) *apg* and (D) spirocyclopentyl.

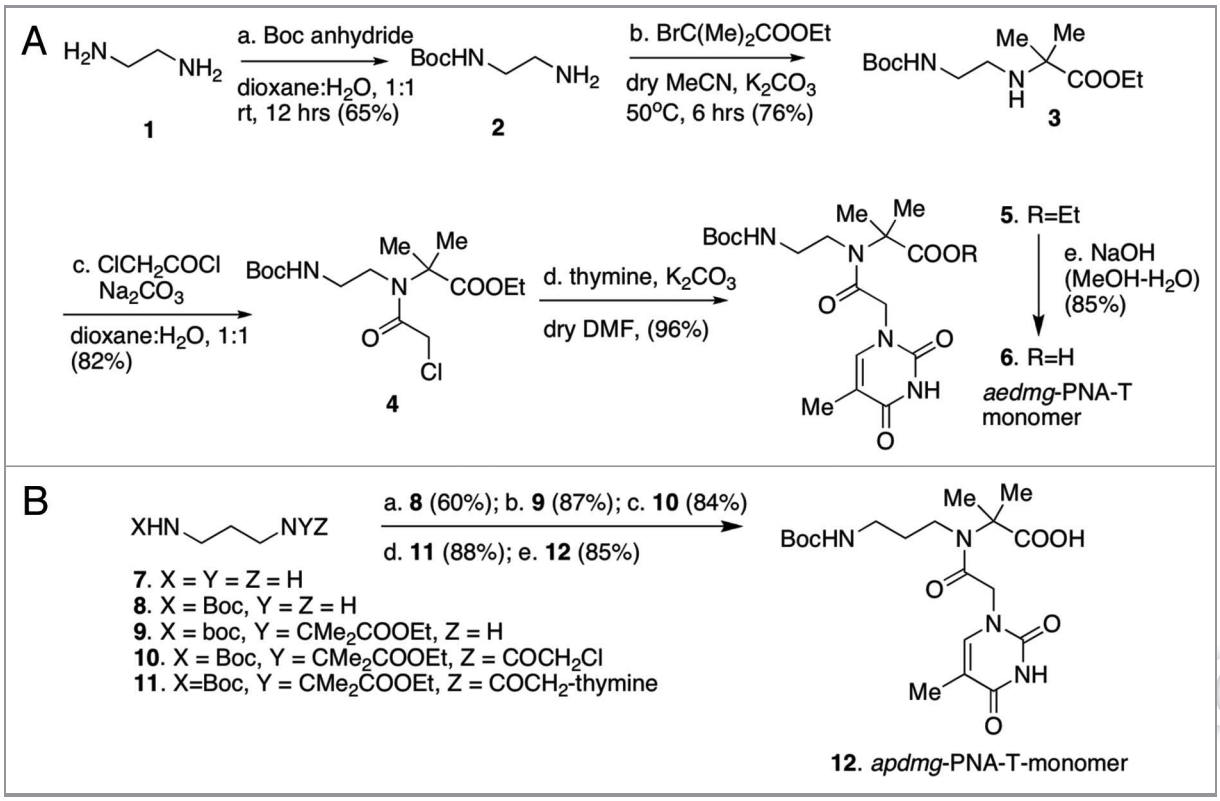

Figure 3. Synthesis of *dmg*-PNA monomers (A) *aedmg*-PNA-T **6** and (B) *apdmg*-PNA-T monomer **12**. Values in brackets indicate yields. In Scheme B reagents a–e are same as in Scheme A.

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.

UV–T_m studies on aedmg-PNA_2:DNA and aedmg-PNA_2:RNA triplexes. The homothymine *aeg*-PNA sequences with

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

Entry	PNA sequence	HPLC Rt	Mol Formula	Mol Wt Calc	Mol. Wt Obs
1.	PNA **1** H–TTTTTTTT-LysNH_2	7.34	$C_{94}H_{127}N_{35}O_{33}$	2275.25	2275.47
2.	PNA **2** H–TTTTTTT**t**-LysNH_2	8.02	$C_{96}H_{131}N_{35}O_{33}$	2303.30	2303.43
3.	PNA **3** H–TTTTT**t**T**t**-LysNH_2	8.32	$C_{98}H_{135}N_{35}O_{33}$	2331.36	2334.60
4.	PNA **4** H–TTT**t**T**t**T**t**-LysNH_2	8.96	$C_{100}H_{139}N_{35}O_{33}$	2359.41	2364.68
5.	PNA **5** H–T**t**T**t**T**t**T**t**-LysNH_2	9.30	$C_{102}H_{143}N_{35}O_{33}$	2387.46	2389.82
6.	PNA **6** H–**tttttttt**-LysNH_2	10.32	$C_{110}H_{159}N_{35}O_{33}$	2499.68	2505.00
7.	PNA **7** H–TTTTTTT***t***-LysNH_2	8.63	$C_{97}H_{133}N_{35}O_{33}$	2317.33	2316.78
8.	PNA **8** H–TTTTT***t***T***t***-LysNH_2	8.78	$C_{100}H_{139}N_{35}O_{33}$	2359.41	2363.74
9.	PNA **9** H–TTT***t***T***t***T***t***-LysNH_2	9.65	$C_{103}H_{145}N_{35}O_{33}$	2401.49	2452.61
10.	PNA **10** H–T***t***T***t***T***t***T***t***-LysNH_2	10.16	$C_{106}H_{151}N_{35}O_{33}$	2443.57	2444.60
11.	PNA **11** H–***tttttttt***-LysNH_2	10.90	$C_{118}H_{175}N_{35}O_{33}$	2611.89	2613.21
12.	PNA **12** H-GTAGATCACT-LysNH_2	10.39	$C_{114}H_{148}N_{60}O_{31}$	2854.81	2860.93
13.	PNA **13** H-G**t**AGA**t**CAC**t**-LysNH_2	13.50	$C_{120}H_{160}N_{60}O_{31}$	2938.97	2941.47
14.	PNA **14** H-G***t***AGA***t***CAC***t***-LysNH_2	14.42	$C_{123}H_{166}N_{60}O_{31}$	2981.05	2978.59

*T aeg, **t** *aedmg* and ***t*** *apdmg* PNA. DNA **15** CGAAAAAAAACG; DNA **16** CGAAAACAAACG . DNA **17** = AGTGATCTAC and DNA **18** = CATCTAGTGA, RNA **19** poly rA, RNA **20** = AGUGAUCUAC and RNA **21** = CAUCUAGUGA. In most PNA oligomers observed Mass correspond to either $M+nH^+$ (n = 1–5) or $M + Na^+$.

cDNA-dA_8 are known to form PNA_2:DNA triplexes.[1,2] The binding stoichiometry of *aedmg*-PNA-T_8:DNA **15** complexes was established as 2:1 expected for a PNA_2:DNA triplex from UV Job's plot (see **Supplemental Material**).[27] The thermal stabilities of triplexes from unmodified PNA **1** and *dmg*-PNA-T_8 (PNA **2–11**) with cDNA **15** and RNA **19** (polyrA_{40}) were determined from temperature-dependent UV absorbance at 260 nm. The UV-T_m plots show a single sigmoidal transition, characteristic of PNA_2: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 T_m values corresponding to peaks from such plots for various PNA_2:DNA and PNA_2:RNA triplexes of *aedmg* and *apdmg*-PNAs with cDNA **5** or RNA **19** are shown in **Table 2**.

It is seen from the UV-melting data (column 1) that introduction of single *aedmg*-PNA-T modification at N-terminus enhanced the T_m of derived PNA_2:DNA **15** triplex 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 triplex. Significantly, the fully modified oligomer (*aedmg*-PNA-T_8 **6**) formed triplex with DNA **15** (entry 6) so strongly that no melting was seen even up to 85°C. A progressive increase in T_m per unit of *aedmg*-PNA-T modification in homothymine sequences suggests that the nature of the structural factors causing stabilization is additive and cumulative.

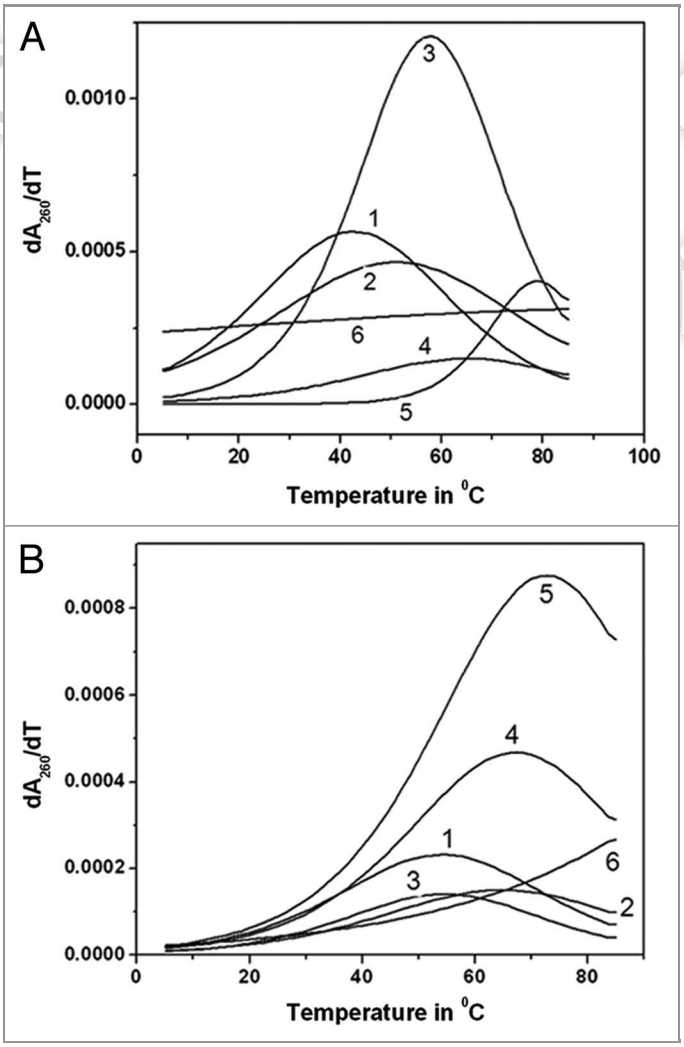

Figure 4. First derivative UV absorbance-temp plots of (A) *aedmg*-PNA_2:DNA and (B) *aedmg*-PNA_2:RNA triplexes. See **Supplemental Material** for corresponding data on *apdmg*-PNA_2:DNA/RNA triplexes. The numbers associated with each curve corresponds to PNAs in **Table 1**.

Since PNA_2:RNA triplexes have slightly different conformational features,[10] the stabilizing effect of the *aedmg*-PNA units on the derived triplexes (PNA_2:RNA **19**) was examined (**Table 1**, column 2). Single N-terminal modification gave only marginal positive effect (ΔT_m = 1.4°C, entry 2) over control triplex (entry 1), while further increase in the number of *aedmg*-PNA units (entry 3–6), gave a T_m enhancement of about 2–2.5°C per modification. Again, PNA $\mathbf{6}_2$:RNA **19** triplex from the fully modified *aedmg*-PNA (entry 6) failed to show complete melting even at 85°C. It was noticed that the melting of *aedmg*-PNA_2:DNA triplexes with PNAs **4** and **5** having three or more modifications had a slightly larger change in hyperchromicity compared with unmodified PNA_2:DNA triplex suggesting that *aedmg*-modified PNAs have better stacking of the bases. It should be pointed out that the T_ms 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 T_m of completely modified *aedmg*-PNA-T_8 (PNA **6**) with DNA **16** having single mismatch in the middle (C instead of A) was measured. A larger destabilization (ΔT_m = -24.4°C, entry 8) was observed for mismatched triplex from fully modified *aedmg*PNA **6** in comparison with analogous triplex from unmodified control PNA 7 (ΔT_m = -12.8°C, entry 7). This not only indicated the formation of triplex by fully modified PNA **6** (although no melting was seen till 85°C) but also confirmed that the stability of *aedmg*-PNA:DNA triplex was not achieved at the expense of loss of sequence selectivity.

The UV-T_m data in **Table 2**, columns 3 and 4 are for the corresponding triplexes derived from *apdmg*–PNA T_8 oligomers, having propylene chain instead of ethylene in the *dmg*-PNA oligomers (PNA **7–11**). While single N-terminal modification resulted in a slight destabilization, further modifications enhanced the T_m by 4.1°C per modification, which is less than 7–9°C seen for triplexes from *aedmg*-PNA (PNA **2–6**). PNA_2: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 *aedmg*-PNA:RNA triplexes. The stabilization of *apdmg*-PNA:DNA/RNA triplexes is interesting, since it was earlier reported that aminopropyl-glycyl PNA[24] destabilized the derived hybrids compared with aminoethylglycyl-PNA. Thus, the *gem*-dimethyl substitution on glycine in both aminoethyl and aminopropyl PNAs stabilize the

Table 2. UV-T_m (°C) of *dmg*-PNA_2:DNA and *dmg*-PNA_2:RNA triplexes*

Entry	PNA sequence	No	t = *aedmg*		No	t = *apdmg*	
	Column No→		1 DNA 15	2 RNA 19		3 DNA 15	4 RNA 19
1.	H–TTTTTTTT–LysNH_2	**1**	43.0	53.8		43.0	53.8
2.	H–TTTTTTT**t**–Lys NH_2	**2**	51.1 (+8.1)	55.2 (+1.4)	**7**	40.3 (-2.7)	44.3 (-9.5)
3.	H–TTTT**t**T**t**–Lys NH_2	**3**	57.9 (+14.9)	64.7 (+10.9)	**8**	48.4 (+5.4)	51.1 (-2.7)
4.	H–TTT**t**T**t**T**t**–Lys NH_2	**4**	64.7 (+21.7)	67.4 (+13.6)	**9**	55.2 (+12.2)	55.2 (+1.4)
5.	H–T**t**T**t**T**t**T**t**–Lys NH_2	**5**	79.6 (+36.6)	72.8 (+19.0)	**10**	59.2 (+16.2)	56.5 (+2.7)
6.	H–**t t t t t t t t**–Lys NH_2	**6**	> 85 (> 43)	> 85 (> 32)	**11**	76.9 (+33.9)	60.6 (+4.1)
7.	H–TTTTTTTT–LysNH_2	**1**†	30.2 (-12.8)				
8.	H–**t t t t t t t t**–Lys NH_2	**6**†	61.6 (-24.4)				

*Experiments were repeated at least thrice and the T_m values were obtained from the peaks in the first derivative plots. T_m values are accurate to (±) 0.5°C. T and **t** indicate *aeg* and *dmg*-PNA respectively. Values in brackets denote degree (ΔT_m) of stabilization with (+) or destabilization (-) compared with control PNA **1**. Numbers in bold represent PNA (see **Table 1** for sequence). †T_m refers to complex with mismatch DNA**16**.

constituted DNA and RNA triplexes over that of triplexes of unmodified PNA and the stabilization is significantly better for DNA triplexes in comparison to RNA triplexes.

UV–T_m studies on aedmg-PNA:DNA/RNA and apdmg-PNA:DNA/RNA duplexes. The PNA:DNA and PNA:RNA duplexes differ in conformation and structural features compared with PNA_2:DNA and PNA_2:RNA triplexes,[10] and hence the mixed base sequence *aeg* (**12**), *aedmg* (**13**) and *apdmg* (**14**) PNAs were examined for their duplex forming abilities with antiparallel (ap) cDNA **17** and RNA **20** sequences (**Fig. 5**). PNAs with mixed base purine-pyrimidine sequences can form duplexes also in parallel orientations (parallel: N-terminus of PNA aligned with 5' of DNA/RNA; antiparallel: N-terminus of PNA aligned with 3'-end of DNA/RNA) and hence this possibility was examined with parallel (p) cDNA **18** and RNA **21** oligonucleotides. 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.

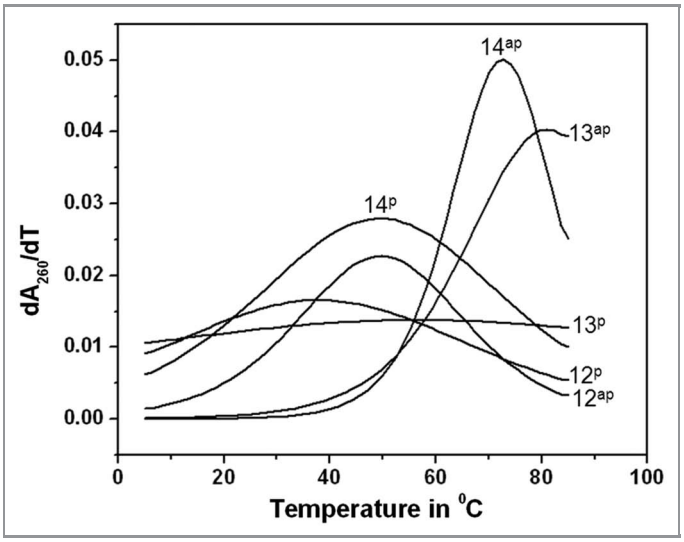

Figure 5. First derivative UV absorbance-temp plots of PNA:DNA duplexes. p and ap refer to parallel and antiparallel duplexes. For PNA numbers see **Table 1**.

The duplexes were constituted by mixing equimolar amounts of *aeg*-PNA **12**, *aedmg*-PNA **13** and *apdmg*-PNA **14**, individually with the cDNA **17**, RNA **20** for antiparallel duplexes and DNA **18**, RNA **21** for parallel PNA:DNA duplexes. The Job's plot completely supported formation of 1:1 duplexes in both *aedmg*-PNA **13** and *apdmg*-PNA **14** complexes with DNA **17** (see **Supplemental Material**). The T_m data (**Table 3**) was obtained from the plot of UV absorbance vs temperature and show that the antiparallel duplex (*aedmg*-PNA **13**:DNA **17**) with 3 modifications exhibits very high stabilization (entry 1, ΔT_m = +31.1°C, 10°C/modification) compared with the corresponding unmodified duplex (PNA **12**:DNA **17**, entry 1). The parallel duplex of modified *aedmg*-PNA (PNA **13**:DNA **18**) is also significantly stabilized (entry 2, ΔT_m = +19°C, 6°C/modification) compared with the unmodified parallel duplex (PNA **12**:DNA **18,** entry 2). *apdmg*-PNA **14** showed enhanced stabilization of its antiparallel duplex with cDNA **17** (entry 1, ΔT_m = +23°C, 7°C/modification) over that of unmodified PNA **12**, but about the same stability for parallel duplex (entry 2).

The mixed unmodified PNA **12** and the modified *aedmg*-/*apdmg*-PNA **13** also forms antiparallel and parallel duplexes with

Table 3. UV-T_m of *dmg*-PNA:DNA and *dmg*-PNA:RNA duplexes*

No	DNA/RNA	PNA 12	*apdmg*-PNA 13	*apdmg*-PNA 14
1	DNA **17** AGTGATCTAC (ap)	49.8	80.9 (+31.1)	72.8 (+23.0)
2	DNA **18** CATCTAGTGA (p)	37.5	56.5 (+19.0)	49.7 (+12.2)
3	RNA **20** AGUGAUCUAC (ap)	50.1	72.3 (+22.2)	64.7 (+14.6)
4	RNA **21** CAUCUAGUGA (p)	40.5	31.6 (-8.9)	32.9 (-7.6)

*T_m 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, $^{t=aeg\_PNA;t=aedmg\_PNA}$-T/*apdmg*-PNA-T, ap and p 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 antiparallel 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 stibilize (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 PNA_2: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-C^{α} and C^{α}-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.[23] *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 backbones 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*-dilkyl 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.

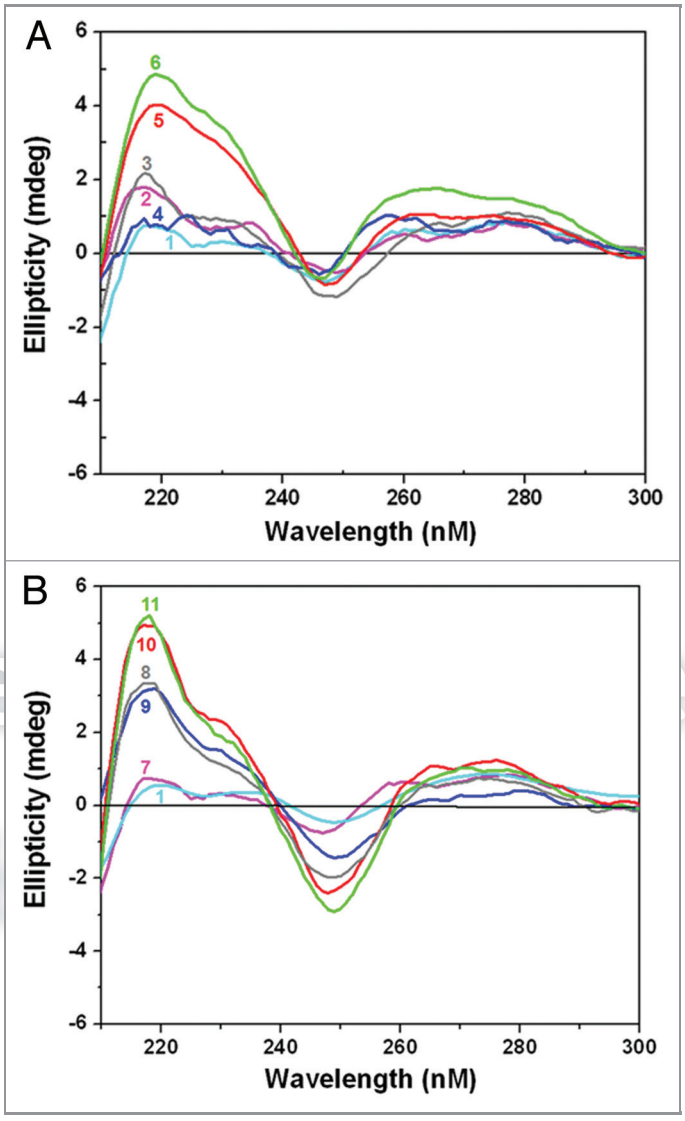

Figure 6. CD spectra of (A) *aedmg*-PNA_2:DNA triplexes and (B) *apdmg*-PNA:DNA triplexes. The number shown above each curve refers to the PNA sequence shown in **Table 1**.

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**

(3.45 g, 63% yield). ^{1}H NMR ($CDCl_3$) δ: 5.21 (br s, 1H, NH), 3.32 (t, 2H, J = 8 Hz), 2.54 (t, 2H, J = 8 Hz), 1.42 [s, 9H, C(CH_3)$_3$]. M_{calc} ($C_7H_{16}N_2O_2$) = 160.21 and M_{obs} = 161.18 (M+1).

***N*1-Boc-1,3-diaminopropane (14).** 1,3-diaminopropane **13** (20 g, 0.27 mol) on reaction with Boc-anhydride (5.9 g, 27 mmol) under identical reaction conditions as above gave a mixture of *N*1,*N*3-di-Boc and the *N*1-mono-Boc derivative **14** from which the former compound was precipitated and removed by filtration. The product obtained by repeated extraction from ethyl acetate and removal of solvents yielded the mono-*N*-Boc-diaminopropane **14** (4 g, 60% yield). ^{1}H NMR ($CDCl_3$) δ: 4.78 (br s, 1H, NH), 3.32 (q, 2H), 2.74 (t, 2H), 1.59 (t, 2H), 1.42 [s, 9H, C(CH_3)$_3$]. M_{calc} ($C_7H_{16}N_2O_2$) = 160.21 and M_{obs} = 174.18 (M+1).

Ethyl *N*–Boc-(aminoethyl)-α,α-dimethylglycinate (3). The *N1*-(Boc)-1,2-diaminoethane **2** (3.2 g, 20 mmol) was treated with 2-bromoethylisobutyrate (3 ml, 20 mmol) in dry acetonitrile (50 ml) in the presence of anhydrous K_2CO_3 (5.5 g, 40 mmol) and the mixture was stirred at 70°C for 15 h. The reaction mixture was concentrated to remove the acetonitrile and work-up with ethylacetate-water and purification by silica gel column chromatography gave ethyl-*N*-Boc-(aminoethyl)-α,α-dimethylglycinate **3** as a yellowish oil (4.2 g, 76.6% yield). ^{1}H NMR ($CDCl_3$) δ: 5.29 (br s, 1H, N*H*), 4.22–4.11 (q, 2H), 3.26–3.21 (t, 2H), 2.7–2.65 (t, 2H), 1.43 [s, 9H, C (CH_3)$_3$], 1.34 [s, 6H, C(CH_3)$_2$], 1.30–1.23 (t, 3H); ^{13}C NMR ($CDCl_3$) δ: 176.8, 171.2, 156.6, 79.7, 62.5, 61.6, 43.9, 37.7, 28.1, 22.1, 21.1 and 13.8. M_{calc}($C_{13}H_{26}N_2O_4$) = 274.36 and M_{obs} = 275.21 ($M+H^+$).

Ethyl *N*-Boc-(aminopropyl)-α,α-dimethylglycinate (9). The *N1*-Boc-1,3-diaminopropane **14** (3.12 g, 18 mmol) was reacted with 2-bromoethylisobutyrate (2.65 ml, 18 mmol) under similar conditions, followed by usual work-up and chromatography gave ethyl *N*-Boc-(aminopropyl)-α,α-dimethylglycinate **15** as yellowish oil (4.5 g, 87% yield). ^{1}H NMR ($CDCl_3$) δ: 5.02 (br s, 1H, N*H*), 4.18–4.07 (q, 2H), 3.17–3.11 (t, 2H), 2.51–2.45 (t, 2H), 1.63–1.56 (t, 2H), 1.40 [s, 9H, C(CH_3)$_3$], 1.26 [s, 6H, C(CH_3)$_2$], 1.23–1.20 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 176.8, 155.9, 60.7, 58.8, 41.9, 38.9, 30.5, 28.3, 25.2, and 14.2. M_{calc} ($C_{14}H_{28}N_2O_4$) = 286.38 and M_{obs} = 289.17 ($M+3H^+$).

Ethyl *N*-(Boc-aminoethyl)-N-(chloroacetyl)-α,α-dimethylglycinate (4). The ethyl *N*-Boc-(aminoethyl)-α,α-dimethylglycinate **3** (4.0 g, 14.6 mmol) was taken in 10% aqueous Na_2CO_3 (75 ml) and dioxane (60 ml). Chloroacetyl chloride (3.5 ml, 43.8 mmol) was added in two portions with vigorous stirring. The reaction was complete within an hour and was brought to pH 8.0 by addition of 10% aqueous Na_2CO_3 and concentrated to remove the dioxane. The product after work-up was purified by column chromatography to obtain the ethyl *N*-Boc-(aminoethyl)-*N*-(chloroacetyl)-α,α-dimethylglycinate **4** as a yellowish oil. (4.2 g, 82% yield). ^{1}H NMR ($CDCl_3$) δ: 5.13 (br s, 1H, N*H*), 4.27–4.17 (q, 2H), 4.13 (s, 2H), 3.57–3.5 (t, 2H), 3.36–3.30 (t, 2H), 1.49 [s, 6H, C(CH_3)$_2$], 1.43 [s, 9H, C(CH_3)$_3$], 1.27–1.20 (t, 3H). ^{13}C NMR ($CDCl_3$) 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_{calc} ($C_{15}H_{27}N_2O_5Cl$) = 350.16 and M_{obs} = 352.01 (M+2).

Ethyl *N*-Boc-(aminopropyl)-N-(chloroacetyl)-α,α-dimethylglycinate (10). The ethyl *N*-Boc-(aminopropyl)-α,α-dimethylglycinate **15** (3.6 g, 12.5 mmol) reacted with chloroacetyl 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). ^{1}H NMR ($CDCl_3$) δ: 4.77 (br s, 1H, N*H*), 4.17–4.06 (q, 2H), 4.04 (s, 2H), 3.48–3.40 (t, 2H), 3.17–3.10 (t, 2H), 1.91–1.82 (t, 2H), 1.46 [s, 6H, C(CH_3)$_2$], 1.43 [s, 9H, C (CH_3)$_3$], 1.23–1.17 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 173.64, 166.22, 156.09, 61.66, 61.05, 42.19, 41.70, 32.33, 28.30, 23.75, and 13.97.M_{calc}($C_{16}H_{29}N_2O_5Cl$) = 364.16 and M_{obs} = 365.13 (M+1).

***N*-(Boc-aminoethyl-α,α-dimethylglycyl)-thymine ethyl ester (5).** Ethyl *N*-Boc-(aminoethyl)-*N*-(chloroacetyl)-α,α-dimethylglycinate **4** (4.0g, 11.4 mmol) was stirred with anhydrous K_2CO_3 (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 purificationgave *N*-Boc-(aminoethyl-α,α-dimethyl glycyl)-thymine ethyl ester **5** (4.8 g, 96.5%). ^{1}H NMR ($CDCl_3$) δ: 6.94 (s, 1H), 5.43 (br s, 1H, N*H*), 4.57 (s, 2H), 4.17–4.06 (q, 2H), 3.57–3.50 (t, 2H), 3.41–3.34 (t, 2H), 1.87 (s, 3H, CH_3), 1.48 [s, 6H, C(CH_3)$_2$], 1.42 [s, 9H, C(CH_3)$_3$], 1.23–1.16 (t, 3H). M_{calc} ($C_{20}H_{32}N_4O_7$) = 440.49 and M_{obs} = 463 ($M+Na^+$).

***N*-Boc-(aminopropyl-α,α-dimethylglycyl)-thymine ethyl ester (11).** Ethyl *N*-Boc-(aminopropyl)-*N*-(chloroacetyl)-α,α-imethylglycinate **16** (2.0 g, 5.5 mmol) was reacted with thymine (0.76 g, 6.1 mmol) under similar conditions in presence of K_2CO_3 in DMF followed by work-up and column chromatographic purification to give *N*-Boc-(aminopropyl)-α,α-dimethyl-glycyl-thymine ethyl ester **17** as white solid (2.2 g, 88% yield). ^{1}H NMR ($CDCl_3$) δ: 6.98 (s, 1H), 4.95 (br s, 1H, N*H*), 4.49 (s, 2H), 4.16–4.06 (q, 2H), 3.49–3.41 (t, 2H), 3.24–3.18 (t, 2H), 1.89 (s, 3H), 1.47 [s, 6H, C (CH_3)$_2$], 1.42 [s, 9H, C(CH_3)$_3$], 1.23–1.16 (t, 3H). ^{13}C NMR ($CDCl_3$) δ: 173.7, 166.4, 164.2, 156.4, 151.2, 110.6, 61.9, 61.19, 41.49, 31.92, 28.39, 23.98, 14.03, 12.32. M_{calc}($C_{21}H_{34}N_4O_7$) = 454.61 and M_{obs} = 477.18 ($M+Na^+$).

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-H^+ 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%), ^{1}H NMR ($CDCl_3$) δ: 10–11 (2H, thymine NH and –COOH), 7.18 (s, 1H), 5.43 (br s, 1H.N*H*), 4.2–4.16 (q, 2H), 3.5–3.56 (t, 2H), 3.39–3.32 (t, 2H), 1.87 (s, 3H, CH_3), 1.48 [s, 6H, C(CH_3)$_2$], 1.42 [s, 9H, C(CH_3)$_3$]. M_{calc} ($C_{18}H_{28}N_4O_7$) = 412.44 and M_{obs} = 435.57 ($M+Na^+$).

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 DMF/DCM and pyridine as a base. The MBHA resin with free amine was functionalized by coupling with N (α)-Boc-N(ε)-Cl$^-$Cbz- L-lysine using DCCI as coupling reagent. The lysine loaded resin was swollen in dry

CH_2Cl_2 (30 min) and treated with 50% TFA in CH_2Cl_2 (1 ml × 2, 15 min each). After washing with CH_2Cl_2, neutralization of TFA salt with 5% DIPEA in CH_2Cl_2 (1 ml × 3, 2 min each) and washed further with CH_2Cl_2. The unmodified/modified PNA monomers were coupled to resin in presence of HBTU/HOBt/DIPEA 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/TFMSA in presence of scavenging 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 TFMSA (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-T_m Measurements. The concentration of PNA and DNA was calculated on the basis of absorbance using the molar extinction coefficients of nucleobases for A = 15.4, t = 8.8, C = 7.3 and G = 11.7 $M^{-1}cm^{-1}$. 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 scanning from 5 to 85°C at a ramp rate of 0.2°C per minute. Experiments were repeated at least thrice and the T_m values were obtained from the peaks in the first derivative plots. The reported T_m values are accurate to (±) 0.5°C.

Conclusions

In summary, the incorporation of gem-dimethyl substituted *aedmg*-PNA monomer into the *aeg*-PNA backbone preferentially increased the T_m of the derived complexes with DNA as compared with RNA. This perhaps arises from a structural preorganization of the backbone due to steric constrains 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[17-19] has shown that the cyclic cyclohexanyl PNAs show strong preference for RNA binding and the present results allow tuning of PNA backbone to DNA binding by introduction of rigidity 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 aminoethyl segment, combining subsitution 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 here:
www.landesbioscience.com/journals/artificialdna/article/19185

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