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In situ, on-resin synthesis of 8-Br/NH_2 adeninyl peptide nucleic acid (PNA) oligomers and complementation studies with DNA

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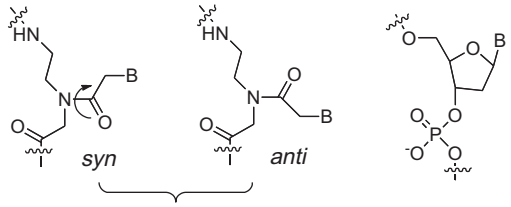
ABSTRACT

A new, novel and efficient in situ synthesis of 8-aminoadeninyl PNA oligomers from corresponding 8-bromoadeninyl PNA oligomers is reported. The study of hybridisation properties of (8-Br/8-NH_2) PNA oligomers with cDNA reveals substitution-site dependent stabilization of derived triplexes and duplexes.

1. Introduction

Peptide nucleic acid (**1**), an interesting class of DNA (**2**) mimic possessing gene targeting properties has emerged as a novel antigene/antisense agent in the field of medicinal chemistry.[1] In PNA, a neutral and achiral polyamide backbone consisting of *N*-(2-aminoethyl) glycyl (aeg) units replace the charged sugar–phosphate backbone of DNA. The nucleobases (A/G/C/T) are attached to the backbone through tertiary acetamide linker and PNA binding to the target DNA/RNA sequences occurs with high sequence specificity and affinity.[2] PNA binds to complementary DNA and RNA to form duplexes via Watson–Crick (WC) base pairs and triplexes through a combination of WC and Hoogsteen (HG) hydrogen bonding.



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PNA:DNA/RNA hybrids exhibit thermal stability higher than that of analogous DNA:DNA and DNA:RNA complexes.[3] Because of this attractive feature and stability to proteases and nucleases, PNAs are of great interest in medicinal chemistry, with potential for development as gene-targeted drugs and as reagents in molecular biology and diagnostics.[4] Some of the drawbacks for therapeutic applications of PNA are their poor aqueous solubility, insufficient cellular uptake and ambiguity in orientation (parallel/antiparallel) selectivity of binding with DNA/RNA.[5]

To address these problems, several PNA analogues have been reported to date with modifications in backbone,[5] side chain or nucleobase[6] which alter the hybridizing properties of the derived PNA:DNA/RNA hybrids. Nucleobase modifications in PNA are very few since structural changes in base may directly affect the WC/HG base pairing in PNA:DNA hybrids. In the literature, 8-oxo/amino substituted purines are known to strengthen the HG base pairing and hence triplex properties through the additional H-bonding possible from the five-membered ring of purines (Fig. 1).[7] We have recently reported the interesting properties of cyanuryl PNAs containing cyanuric acid having additional H-bond donors and acceptors in place of thymine.[8a]

8-Aminoadenosine was earlier shown to induce reverse HG base pairing with 2′-deoxythymidine (Fig. 1a, **3**) arising from the extra H-bonding of 8-amino group, in preference to WC base pairing, as proved from ^{1}H NMR study of 8-amino-dA:dT complex.[8b] Upon introducing 8-amino-dA into DNA oligomer, an increase in the stability of triplexes was observed[9] because of the possibility of three hydrogen bonds from HG side (five-membered ring of purine) without affecting the normal WC H-bonding (Fig. 1b).

8-Aminopurine has also been shown to increase the stability of DNA hairpins.[10] Binding of polypyrimidine oligonucleotide single strands by modified clamps containing 8-aminopurines results in the formation of very stable antiparallel triplexes at room temperature.[9b] In DNA, purines A and G having substituent at 8-position

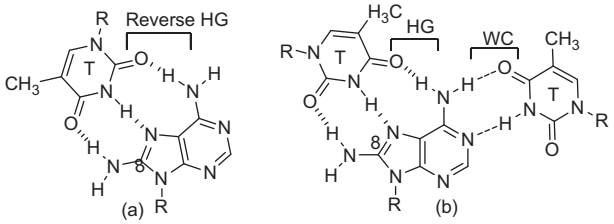

Figure 1. Structures of (a) dT:8-NH_2-A base pair **4**, (b) dT:8-NH_2-A:dT base triad **3**.

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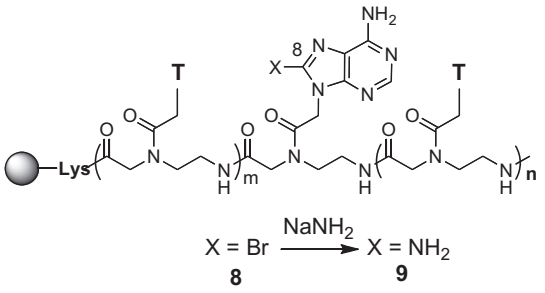
tend to adopt *syn* conformation at the glycosidic bond due to steric effects (in contrast to the *anti* conformation in 8-unsubstituted purines) which influences the hybridization strengths.[11] Such effects may not be of relevance in PNA which is non-glycosidic. However, restriction around the tertiary amide bond in PNA generates rotamers (**1**, *syn* and *anti*) and since the base is not directly linked to the amide, the steric constraints may not be that significant. It would, therefore, be interesting to study the effect of incorporating 8-substituted purine in PNAs on their complementation properties with DNA. This manuscript reports on a novel in situ, on-resin strategy for synthesis of hitherto unknown 8-aminoadenine PNAs from 8-bromoadenine PNAs and preliminary biophysical studies of 8-bromo/amino adenine PNA containing PNA:DNA duplexes.



Scheme 2. In situ on-resin conversion of 8-bromoadeninyl PNA into 8-aminoadeninyl PNA oligomer. The reaction monitored by HPLC (Fig. 2).

2. Results

2.1. Preparation of 8-bromoadeninyl PNA monomer 7

The direct introduction of 8-aminoadenine PNA monomer into oligomer necessitates the use of a suitable protecting group for 8-NH_2 function. An ideal synthetic precursor for 8-aminoadenine is 8-bromoadenine, which can be transformed into the target 8-aminopurine by treatment with saturated ammonia in methanol or dioxane.[12] The starting material adeninyl aeg PNA monomer **5** was made by literature procedures[13] starting from ethylenediamine. Compound **5** was brominated (Scheme 1) by treatment with molecular bromine in dioxane and 10% aq Na_2HPO_4 to yield the required 8-bromoadeninyl compound **6** in moderate yield (55%). Attempts to convert the 8-bromo derivatives directly to 8-amino analogues by reacting directly with ammonia in dioxane failed and instead gave the carboxamide from a reaction of ester function. Hence the ester **6** was hydrolysed by alkali to yield 8-bromoadeninyl PNA monomer **7** that was directly used for coupling in solid phase synthesis to make 8-bromo PNA oligomers.

2.2. Conversion of 8-bromoadeninyl PNA oligomer 8 to 8-aminoadeninyl PNA oligomer 9

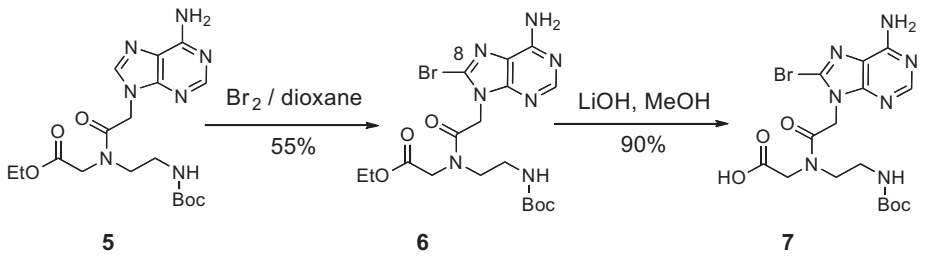
The protecting group selection for the 8-amino group in 8-aminoadenine was found to be difficult, since the conventional benzoyl group could not be deprotected under standard conditions. In DNA synthesis, the amino function at C-8 was earlier protected as the acid-labile dimethylamino formamidine.[9c] However, this group is not stable under standard protocols of solid phase synthesis of PNA, where 50% TFA in DCM is used for the deprotection of 'Boc' group. Due to these problems in finding a suitable protecting group for amino function in 8-aminoadenine at monomer level, the direct conversion of 8-bromoadeninyl PNA oligomers **8** to 8-aminoadeninyl PNA oligomers **9** on resin was attempted (Scheme 2).

8-Bromoadeninyl PNA monomer **7** was incorporated at specific sites (Table 1) into PNA-T_8 oligomer **8** during solid support synthesis on MBHA resin using standard protocols.[14] After the completion of synthesis, before deprotection the PNA oligomers containing 8-bromoadenine **8** were individually reacted on solid support with sodamide/DMF under reflux conditions to yield the corresponding 8-aminoadeninyl PNA oligomers **9**. The addition of silver fluoride was found to electrophilically assist the removal of bromine by silver in amination reaction and also improve the yields. The progress of amination reaction was monitored through HPLC (Fig. 2) shown for a typical reaction of the oligomer PNA **15** into PNA **19** cleaved from the solid support at different intervals using trifluoromethane sulphonic acid (TFMSA) in TFA.[1b] The reaction was found to be complete in about 15 h when the product, 8-aminoadenine PNA oligomer appeared as single peak at a longer R_t value compared to that of 8-bromoadenine PNA oligomer in HPLC. The product was characterized by MALDI-TOF spectroscopy as the 8-amino adenine PNA oligomer **19**.

The other 8-aminoadenine PNA oligomers **20**–**22** were prepared by a similar protocol from the corresponding 8-bromoadeninyl PNA oligomers **16**–**18**. In case of the synthesis of the mixed base purine-rich 8-aminoadenine PNA oligomer **22**, use of silver fluoride led to lower yields, presumably due to metal complexation of the purine bases. All synthesized PNA oligomers **15**–**24** were individually cleaved from the resin by TFMSA-TFA method, purified through gel filtration column followed by RP HPLC and characterized by MALDI-TOF spectrometry (Table 1). Generally, the HPLC retention time (R_t) value of 8-bromoadeninyl PNAs (PNA **15**–**18**) was between 7.7 and 8.1 min, while the corresponding 8-aminoadeninyl PNAs (PNA **19**–**22**) eluted in 8.9–9.35 min range.

2.3. Biophysical studies of 8-bromo/aminoadeninyl PNA:DNA complexes

The T_m values of the triplexes from various PNAs hybridized with complementary DNA **25** and **26** were determined from temperature-dependent UV absorbance data (Fig. 3). The binding stoichiometry was determined from Job plot generated from CD data of various relative compositions of PNA and DNA and was found to be 2:1 for homooligomeric PNA:DNA complexes indicating the formation of PNA_2:DNA triplexes. It should be pointed out that each triplex with single modification per PNA chain actually



Scheme 1. Synthesis of 8-bromoadeninyl monomer **7**.

Table 1
MALDI-TOF spectral data of synthesized PNAs*

Entry	Sequence	Molecular formula	Mol wt Calcd	Mol wt Obsd
PNA **10**	H-**A**TTTTTTT-*Lys*	$C_{94}H_{126}N_{38}O_{31}$	2284.3	2281.3
PNA **11**	H-TTTTTTT**A**-*Lys*	$C_{94}H_{126}N_{38}O_{31}$	2284.3	2284.8
PNA **12**	H-TTTT**A**TTT-*Lys*	$C_{94}H_{126}N_{38}O_{31}$	2284.3	2305.3
PNA **13**	H-**A**TTT**A**TTT-*Lys*	$C_{94}H_{125}N_{41}O_{29}$	2293.3	2293.1
PNA **14**	H-GT**A**G**A**TC**A**CT-*Lys*	$C_{114}H_{148}N_{60}O_{31}$	2854.9	2859.3
PNA **15**	H-***A***TTTTTTT-*Lys*	$C_{94}H_{125}BrN_{38}O_{31}$	2360.9	2363.9
PNA **16**	H-TTTTTTT***A***-*Lys*	$C_{94}H_{125}BrN_{38}O_{31}$	2360.9	2364.7
PNA **17**	H-TTTT***A***TTT-*Lys*	$C_{94}H_{125}BrN_{38}O_{31}$	2360.9	2364.2
PNA **18**	H-***A***TTT***A***TTT-*Lys*	$C_{94}H_{123}Br_2N_{41}O_{29}$	2447.8	2452.8
PNA **19**	H-**a**TTTTTTT-*Lys*	$C_{94}H_{127}N_{39}O_{31}$	2282.9	2300.8
PNA **20**	H-TTTTTTT**a**-*Lys*	$C_{94}H_{127}N_{39}O_{31}$	2297.9	2297.7
PNA **21**	H-TTTT**a**TTT-*Lys*	$C_{94}H_{127}N_{39}O_{31}$	2297.9	2299.0
PNA **22**	H-**a**TTT**a**TTT-*Lys*	$C_{94}H_{127}N_{43}O_{29}$	2322.0	2320.9
PNA **23**	H-GT***A***G***A***TC***A***CT-*Lys*	$C_{114}H_{145}Br_3N_{60}O_{31}$	3089.8	3093.7
PNA **24**	H-GT**a**G**a**TC**a**CT-*Lys*	$C_{114}H_{151}N_{63}O_{31}$	2897.6	2928.7

* A = Adenine, ***A*** = 8-bromoadenine, **a** = 8-aminoadenine, PNA **12**, Obsd Mol wt corresponds to ($M+Na^+$), Mol wts were calculated by Chem Draw software. Differences of 4–5 units or lower arise due to observation of $M+H^+/M+2H^+$ in some cases due to presence of NH_2 groups and fractional weight differences due to isotopes.

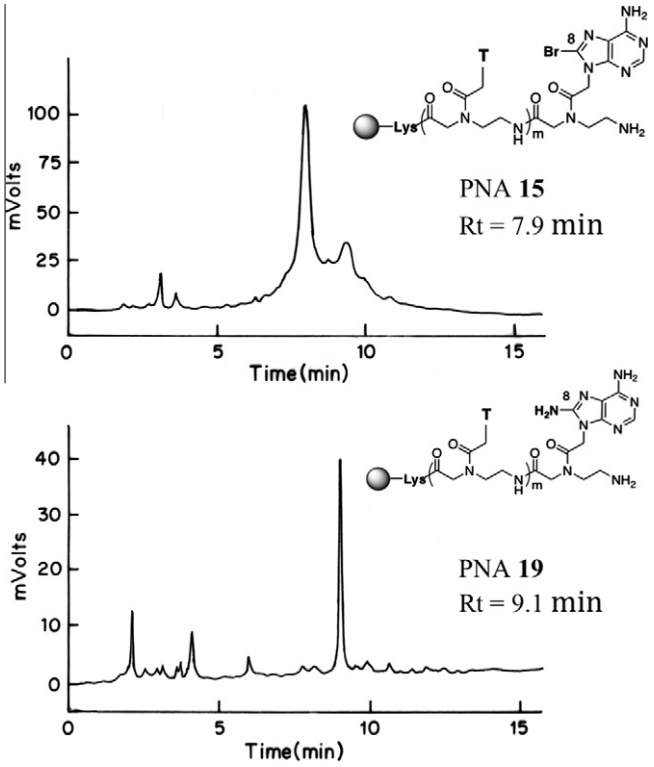


Figure 2. Crude HPLC profiles of 8-bromoadeninyl PNA **15** corresponding 8-aminoadeninyl PNA (i) and (ii) PNA **19**.

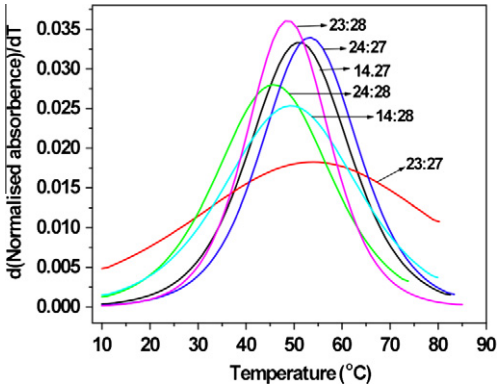


Figure 3. UV–T_m first derivative curves of duplexes of adeninyl **14**, 8-bromoadeninyl **23** and 8-aminoadeninyl **24** PNAs with the corresponding complementary *ap*/*p* DNA.

corresponds to two modifications per triplex. Antiparallel orientation corresponds to C-terminus of PNA facing the 5′-terminus of DNA and parallel orientation refers with C-terminus facing 3′-end of DNA. The cationic lysine was introduced at C-terminus in all PNAs to improve the aqueous solubility. In a PNA_2:DNA triplex, one PNA strand binds DNA in antiparallel orientation with WC pairing and the other PNA strand in parallel orientation binds DNA in HG mode.

The T_m values for different triplexes and duplexes are given in Table 2. The replacement of single T in PNA-T_8 by A at N-terminus (PNA **10**, AT_7) or C-terminus (PNA **11**, T_7A) was found to stabilize the derived PNA_2:DNA(CGA_7TCG) triplexes over unmodified control triplex ($(PNA\text{-}T_8)_2$:DNA(CGA_8CG) by 16–20 °C. (entry 1, column 1). The 8-bromoadeninyl PNA oligomers PNA **15** and PNA **16** with N- and C-terminus modifications stabilized the corresponding triplexes by 8.2 °C (entry 1, column 2) and 4.7 °C (entry 2, column 2), respectively, over the triplexes formed by PNA **10** and PNA **11**. The oligomer PNA **18** with simultaneous modifications with A (8-bromoadenine) at N-terminus and in centre enhanced the T_m of triplex by 7.7 °C (entry 3, column 2) suggesting a slight destabilization component due to modification in centre. In comparison, 8-aminoadeninyl PNA oligomers **19**–**22** showed interesting variations depending on modification site. While PNA **20** with C-terminus modification (entry 2, column 3) stabilized the triplexes by 9.3 °C, the N-terminus modification PNA **19** (entry 1, column 3) considerably destabilized the triplex by 25 °C. Consequently, PNA **22** with double 8-aminoadenine modifications at N-terminus and middle (entry 3, column 3) destabilized the triplex by only 6.5 °C. Since N-terminus modification induced larger destabilization, this suggests a real positive contribution to stability by middle modification.

The sequence specificity of PNA triplex hybridizations was examined through the study of melting properties of PNAs **11**, **16** and **20** with single mismatch containing DNA **29** (SI, S19). The control PNA **11** formed triplex with mismatch DNA sequence with T_m lower by 8.0 °C, while both C-terminus substituted 8-bromo and 8-aminoadeninyl PNAs **16** and **20** exhibited destabilization by 8.5 °C, as good as the control sequence PNA. These further confirm the formation of triplexes in all cases.

The relative differences in the effects of N-terminus and middle modifications among the 8-bromo and 8-aminoadenine PNA oligo-

Table 2
UV–T_m of adeninyl, 8-Br/NH_2-adeninyl PNA:DNA triplexes*

No.		Column no. →	1	2	3
		Sequence X=	**A**	***A***	**a**
1	WC-*ap*	*Lys*-TTTTTTT**X**-H	**10**	**15**	**19**
	DNA **25**	5′-GCAAAAAAATCG-3′	60.0	68.2	35.0
	HG-*p*	H-**X**TTTTTTT-*Lys*		(+8.2)	(−25.0)
2	WC-*ap*	*Lys*-**X**TTTTTTT-H	**11**	**16**	**20**
	DNA **25**	5′-GCAAAAAAATCG-3′	65.5	70.2	74.8
	HG-*p*	H-TTTTTTT**X**-*Lys*		(+4.7)	(+9.3)
3	WC-*ap*	*Lys*-TTT**X**TTT**X**-H	**13**	**18**	**22**
	DNA **26**	5′-GCAAATAAATCG-3′	56.0	63.7	49.5
	HG-*p*	H-**X**TTT**X**TTT-*Lys*		(+7.7)	(−6.5)
4		H-GT**X**G**X**TC**X**CT-*Lys*	**14**	**23**	**24**
	DNA **27**	5′CATCTAGTGA-3′	51.3	53.3	52.8
				(+2.0)	(+1.5)
5		H-GT**X**G**X**TC**X**CT-*Lys*	**14**	**23**	**24**
	DNA **28**	3′CATCTAGTGA-5′	48.6	49.7	46.1
				(+1.1)	(−2.5)

* T_m of (PNA T_8-*Lys*)$_2$: DNA (CG:A_8GC) = 44 °C. *Lys* is at C-terminus of PNA. **A** = adenine, ***A*** = 8-bromoadenine, **a** = 8-aminoadenine. All T_m values are an average of three experiments and accurate within ±0.5°. Buffer: sodium phosphate (10 mM), and NaCl (100 mM), pH 7.2.

mers on T_m of their hybrids are interesting. The bulky size of 8-Br substituent in PNA does not destabilize its triplexes, while the smaller size 8-NH_2 substituent actually lowers the T_m, although one expected stabilization by additional H-bonding. This may perhaps occur due to different terminal base stacking patterns induced by these modifications. Further, the designed sequences generate T:T mismatches in WC and HG base pairing, which may contribute to some destabilization. The A:A self base pairing is still permissible in both HG (purine motif) and WC modes contributing to some stability. The net changes in T_m is, therefore, a combination of various sequence effects including the true contributions from C8-substituents.

The mixed purine–pyrimidine PNA sequence **14** and its analogues **23** and **24** having 8-bromo and 8-aminoadenine in place of adenine were examined for the stability of derived PNA:DNA duplexes. PNA **24** having three 8-aminoadeninyl units was synthesized by direct amination of solid supported 8-bromoadeninyl PNA oligomer **23**. DNA sequences **27** and **28** were used for constituting the antiparallel and parallel PNA:DNA duplexes, respectively. The UV–T_m profile of these duplexes is shown in Figure 3.

Table 2 (entry 4 and 5) shows the T_m of mixed base PNA:DNA duplexes. It is seen that antiparallel duplexes of 8-bromo (PNA **23**) and 8-aminoadenine (PNA **24**) PNAs (entry 4, columns 2,3) are more stabilised compared to the control (PNA **14**:DNA **27**, column 1) duplex by 2° and 1.5°, respectively. In parallel duplexes (entry 5), 8-bromoadenine PNA **14**:DNA **28** duplex was slightly more stable (1.1°, column 2) compared to control (PNA **14**:DNA **28**, column 1) while the 8-aminoadenine duplex (PNA **24**:DNA **28**) was destabilised by 2.5° (column 3). Overall, the 8-bromoadenine PNA:DNA duplexes (**23**:**27** and **23**:**28**) were slightly more stable than the 8-amino PNA:DNA duplexes (**24**:**27** and **24**:**28**) in both antiparallel and parallel orientation. However, the 8-aminoadeninyl PNA oligomer **24** exhibited a better discrimination of parallel and antiparallel duplexes (5.0°) compared 8-bromoadenine (3.6°) or unmodified adenine (2.7°). Thus although 8-bromo/amino adeninyl modifications in PNAs did not significantly enhance the complementary binding with DNA in duplexes, they are well tolerated to discriminate the parallel and antiparallel sequences better than the adenine PNA **14**.

Theoretical studies on introduction of the electron donating groups like hydroxyl or amino group at C8-position of adenine[9b] have suggested that these substituents enhance the H-bond accepting potential of N-7, through delocalization of lone pair electrons on 8-NH_2 group (Fig. 4). This is clearly reflected in N^{15} chemical shifts of N^7 of 8-aminoadenine.[15] 8-Oxoadenine stabilizes the triplex with GC base pair of double stranded DNA even at neutral and basic pH.[16] In context of present results on duplex and triplex stabilities of 8-substituted adeninyl PNAs, the relative combined contribution of the steric and electronic effects due to C8-substituents Br and NH_2 groups in modulating the ability of complex formation with DNA assume importance. Introduction of additional hydrogen bonding sites on nucleobases can lead to interesting tolerance of triad combinations in triplexes as reported by us earlier with 5-aminouracil which is a purine mimic.[17]

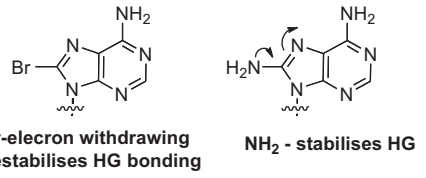


Figure 4. C8-substituent effects on HG bonding stability.

3. Conclusions

The synthetic problem of introducing 8-NH_2 function into adenine in PNAs was overcome through a novel on-resin conversion of 8-bromoadeninyl PNA into 8-aminoadeninyl PNA oligomer. Multiple units of 8-aminoadenines can be simultaneously generated in PNA by this method. The modified PNA oligomers remarkably stabilize the derived triplexes and marginally stabilize the duplexes with DNA. The on-resin conversion method reported here has potential to synthesise 8-guanidino-adeninyl and 8-amino (alkylamino)-guaninyl PNA oligomers and for introduction of substituted functional groups at C8. Such work is in progress.

4. Supplementary material

^1H and ^{13}C NMR spectra of compound **6**, HPLC profiles of 8-bromoadeninyl PNA to 8-aminoadeninyl PNA coversion and PNAs **17**–**20** and MALDI-TOF MS of PNA **17**–**20**, UV–T_m profiles. See doi: 10.1039/ b000000x/.

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14. Synthesis was carried out on MBHA resin (0.2 mmol) using the PNA monomers A/G/C/T (0.05 M) for coupling; For amination the solid supported 8-bromoadeninyl PNA oligomer (10 mg, 0.025 mmol) was treated with AgF (31.7 mg, 0.25 mmol) and $NaNH_2$ (19 mg, 0.27 mmol) in DMF (2 ml) at reflux for 15 h, followed by evaporation under reduced pressure.
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