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Increased DNA binding and sequence discrimination of PNA oligomers containing 2,6-diaminopurine

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ABSTRACT

The synthesis of a diaminopurine PNA monomer, *N*-[*N*^{6}-(benzyloxycarbonyl)-2,6-diaminopurine-9-yl] acetyl-*N*-(2-t-butyloxycarbonylaminoethyl)glycine, and the incorporation of this monomer into PNA oligomers are described. Substitution of adenine by diaminopurine in PNA oligomers increased the T_m of duplexes formed with complementary DNA, RNA or PNA by 2.5–6.5°C per diaminopurine. Furthermore, discrimination against mismatches facing the diaminopurine in the hybridizing oligomer is improved. Finally, a homopurine decamer PNA containing six diaminopurines is shown to form a (gel shift) stable strand displacement complex with a target in a 246 bp double-stranded DNA fragment.

INTRODUCTION

Peptide nucleic acid (PNA) is a nucleic acid mimic in which the entire phosphodiester backbone has been replaced by a pseudopeptide (polyamide). This backbone consists of repeating units of aminoethylglycine with the nucleobases attached through a methylene carbonyl linker. It has been shown that PNA oligomers form highly stable complexes with complementary oligonucleotides (1–4). Mixed purine/pyrimidine oligomers of PNA form 1:1 complexes with Watson–Crick complementary DNA, RNA or PNA oligomers (4), whereas homopyrimidine PNA oligomers bind in a 2:1 (PNA:nucleic acid) mode (1–3,5). PNA has attracted broad attention in the fields of medicinal and bioorganic chemistry as well as in molecular biology and the chemistry and biological and physical properties of PNA were recently reviewed (6–9).

2,6-Diaminopurine (D) is a naturally occurring nucleobase found in, for example, the cyanophage S-2L, where it extensively replaces adenine (10). Since a diaminopurine-thymine base pair resembles a guanine-cytosine base pair (Fig. 1) in terms of both number of hydrogen bonds (three) and extent of interbase pair stacking overlap, duplex stabilization by D-T base pairs is expected. It has been shown that incorporation of diaminopurine into short DNA oligomers increases the thermal stability of the duplex by 0–2°C per D-T base pair (11). It has also been shown that the sequence specificity was improved in some cases (12). Thus a similar increased affinity and specificity upon incorporation of diaminopurine could be expected for PNA–nucleic acid complexes.

We report the synthesis of a diaminopurine monomer (*N*-[*N*^{6}-(benzyloxycarbonyl)-2, 6-diaminopurine-9-yl] acetyl-*N*-(2-t-butyloxycarbonylaminoethyl)glycine) and its incorporation into PNA oligomers. In addition, we show that incorporation of a D-T base pair into a PNA–NA heteroduplex increases the T_m by 2.5–6.5°C per A→D substitution. Furthermore, we show that the sequence specificity can be increased upon incorporation of a diaminopurine nucleobase in PNA.

Finally, it is demonstrated that a homopurine decamer PNA containing six diaminopurines (and four guanines) forms a strand displacement complex with a double-stranded DNA target and that this complex has sufficient stability to be analyzed by band shift assay.

MATERIAL AND METHODS

The A, C, G and T PNA monomers and *N*-(2-bocaminoethyl) glycine were purchased from Perseptive Biosystems. 2,6-Diaminopurine (98%) and standard chemicals were obtained from Aldrich. The phosphoramidites used for DNA synthesis were obtained from CruaChem and were incorporated into DNA oligomers according to standard protocols on a MilliGen/Biosearch 8700 DNA synthesizer. The *N*^{2},*N*^{6}-diphenoxyacetyl-2,6-diaminopurine phosphoramidite was prepared according to the literature (11). RNA oligomers were purchased from DNA Technology and were used as received. Melting points are uncorrected. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). ^{1}H and ^{13}C NMR spectra were obtained in DMSO-d_6 using a Varian 400 MHz Unity or a Bruker 250 MHz AMX spectrometer. Chemical shifts were measured relative to solvent signals (DMSO-d_6: δ_H = 2.50 p.p.m.; δ_{C13} = 39.5 p.p.m.). FAB mass spectra were recorded on a Jeol Hx110/110 mass spectrometer operating in the positive ion mode. MALDI-TOF mass spectra of PNA oligomers were recorded on a Kratos Compact MALDI II instrument operating in the positive ion mode and using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. HPLC was carried out on a 3.9 × 150 mm C-18 Delta Pak 5 µm 100 Å column (Waters). Eluant A: 99.9% H_2O, 0.1% TFA; eluant B: 10% H_2O, 89.9% CH_3CN, 0.1% TFA; a linear gradient of 0–50% eluant B over 35 min at a flow rate of 1.0 ml/min monitored

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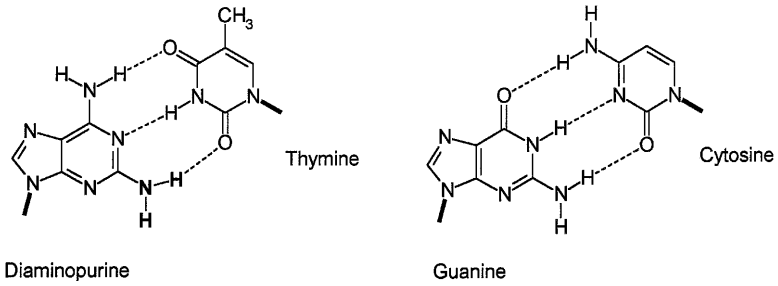
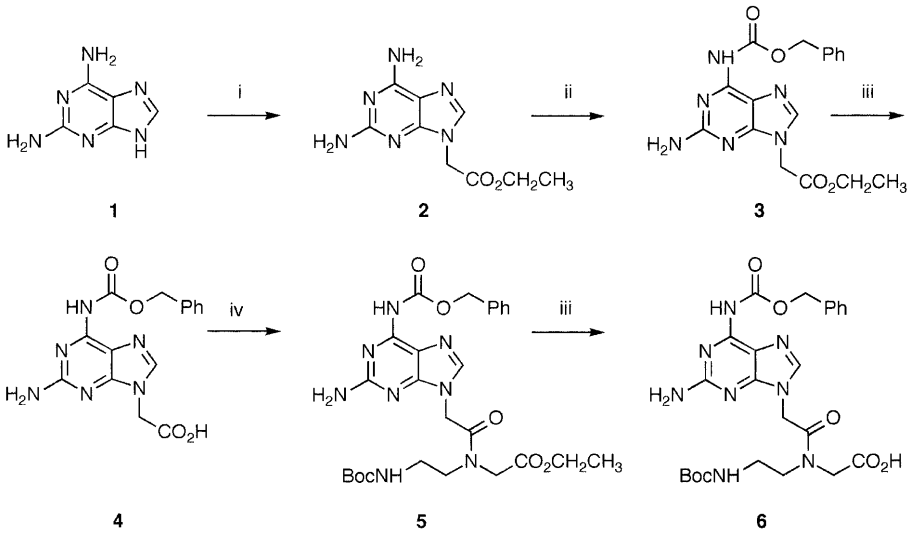


Figure 1. Chemical structures of diaminopurine-thymine and guanine-cytosine base pairs. The amino group that differentiates diaminopurine from adenine is shown in bold.



Scheme 1. (i) NaH, ethyl bromoacetate; (ii) *N*-benzyloxycarbonyl-*N′*-methylimidazolium triflate; (iii) NaOH then HCl; (iv) DCC, DhbtOH, ethyl *N*-(2-bocamino-ethyl)glycinate.

at 260 nm. The column was heated to 50°C. The flow rate was 4.0 ml/min for preparative HPLC.

The PNAs were synthesized according to a published protocol (13) except for one modification: following incorporation of the first 2,6-diaminopurine monomer the usual steps of capping unreacted amines were omitted. Syntheses typically gave a crude product of >90% purity as judged by reverse phase HPLC. The PNAs were purified by reverse phase HPLC and were characterized by MALDI-TOF mass spectrometry. All PNAs showed the expected mass within 5 m.u. (0.2%).

T_m measurements

Absorbance versus temperature was measured at 260 nm using a Gilford Response spectrophotometer. Heating was performed in 0.5°C steps from 5 to 90°C (~0.7°C/min). PNA oligomers were hybridized with complementary DNA sequences in a medium salt buffer containing 100 mM NaCl, 10 mM sodium phosphate and 0.1 mM EDTA, pH 7.0. The samples were heated to 90°C for 5 min, slowly cooled to 20°C and left at 4°C for at least 30 min prior to T_m measurements. The concentrations of all oligomers were determined using UV spectroscopy. The following molar extinction coefficients where used (λ = 260 nm): A, 15.4×10^3; C, 7.3×10^3; G, 11.7×10^3; T, 8.8×10^3; 2,6-diaminopurine, 7.7×10^3 $cm^{-1}M^{-1}$.

Chemical probing

The 246 bp *Eco*RI–*Pvu*II fragment of a pUC19-derived plasmid containing a 5′-GATCGAGAGGAAAA target cloned into the *Bam*HI site was used for gel shift analyses and chemical probing. The plasmid DNA fragment was labeled with ^{32}P ([α-^{32}P]dATP/Klenow DNA polymerase fragment) at the 3′ *Eco*RI site. The ^{32}P 3′-end-labelled *Eco*RI–*Pvu*II fragment was incubated with the desired amount of PNA for 1 h in 100 µl 10 mM Tris–HCl, 1 mM EDTA, pH 7.2, at room temperature (RT) and analyzed by gel electrophoresis in 5% polyacrylamide gels run in TBE (45 mM Tris–borate, 1 mM EDTA, pH 8.5) buffer (gel shift) or by chemical probing with permanganate or diethylpyrocarbonate. Probing with diethylpyrocarbonate (5 µl) was performed for 5 min at RT (with occasional vigorous mixing) and probing with $KMnO_4$ (1 mM final concentration) was performed for 15 s at RT. Reactions were detected as DNA strand breaks following piperidine treatment (10% piperidine at 90°C for 5 min) (cf. 14).The DNA samples were analyzed by high resolution polyacrylamide gel electrophoresis and autoradiography.

Synthesis of the 2,6-diaminopurine PNA monomer

*Ethyl-2,6-diaminopurin-9-ylacetate (**2**).* To a suspension of finely ground 2,6-diaminopurine (**1**) (10.0 g, 66.6 mmol) in dry, degassed DMF (400 ml) was added portionwise NaH (2.93 g, 60% in oil,

73 mmol). After 2 h neat ethyl bromoacetate (14.46 g, 86.6 mmol) was added and the reaction stirred for a further 90 min. The reaction was then filtered with the aid of celite and the clear orange solution was reduced in volume to ~100 ml. Absolute EtOH (100 ml) was added and the solution held at –5°C overnight, which produced light yellow crystals (10.25 g). Recrystallization from EtOH afforded the title compound (**2**) as a cream crystalline solid (5.50 g, 35%). Mp 168–169°C; ^{1}H NMR: δ 7.67 (s, 1H), 6.73 (br s 2H), 5.84 (br s, 2H), 4.83 (s, 2H), 4.13 (q, *J* = 7.2 Hz, 2H), 1.18 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR: δ 168.3, 160.5, 156.1, 152.0, 137.9, 112.72, 61.3, 43.5, 14.1; MS FAB+ 237.1 $(M+H)^+$.

N-Benzyloxycarbonyl-N′-methylimidazolium triflate. This was prepared according to a procedure kindly provided by Dr James Coull (personal communication). Briefly, methyl trifluoromethylsulfonate (29.8 g, 182 mmol) was slowly (20 min) added to a cold (0°C) solution of benzyloxycarbonylimidazole (15) (38.8 g 191 mmol) in DCM (85 ml). Subsequently, the title compound was precipitated by addition of ether (30 ml), isolated by filtration and washed with ether, which yielded 48.7 g (70%) of a white solid, which is stable for at least 3 months when stored dry and cold (–20°C).

*Ethyl-2-amino-6-(benzyloxycarbonylamino)purin-9-ylacetate (**3**).* To a solution of ethyl-2,6-diaminopurin-9-ylacetate (**2**) (7.00 g, 29.6 mmol) in dry degassed 1,4-dioxane (250 ml) was added freshly prepared *N*-benzyloxycarbonyl-*N′*-methylimidazolium triflate (16.30 g, 44.5 mmol) and the reaction stirred for 20 h. The dioxane was removed *in vacuo* and the yellow viscous oil triturated with EtOAc/Et_2O (1:4) until a solid product resulted. Crystallization from 5% MeOH/EtOAc afforded **3** as a light yellow solid (9.60 g, 88%). Mp 185–187°C; ^{1}H NMR: δ 10.12 (br s, 1H), 7.92 (s, 1H), 7.43 (m, 5H), 6.40 (br s, 2H), 5.17 (s, 2H), 4.94 (s, 2H), 4.18 (q, *J* = 7.2 Hz, 2H), 1.21 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR: δ 167.8, 159.9, 154.1, 152.1, 149.8, 140.6, 136.4, 128.2, 127.7, 127.6, 116.7, 65.9, 61.2, 43.5, 13.9; MS (FAB^+) *m/e* calculated for $C_{17}H_{19}N_6O_4$, 371.1468; found, 371.1468 $(M+H)^+$.

Table 1. Thermal stabilities (T_m) of duplexes between PNA or DNA oligomers incorporating 2,6-diaminopurine (D) and complementary DNA, RNA or PNA targets[a,b]

Oligomer		Thermal stability (T_m, °C) of duplexes					
		DNA target[c]		RNA target[d]		PNA target[e]	
		T_m	ΔT_m/base	T_m	ΔT_m/base	T_m	ΔT_m/base
H-GTA GAT CAC T-$LysNH_2$	**I**	51.0		55.0		67.0	
H-GTA G**D**T CAC T-$LysNH_2$	**II**	56.0	5.0	59.0	4.0	71.5	4.5
H-GT**D** G**D**T C**D**C T-$LysNH_2$	**III**	67.0	5.3	68.5	4.5	81.0	4.5
H-AGT GAT CTA C-$LysNH_2$	**IV**	49.0		53.5		67.0	
H-AGT G**D**T CTA C-$LysNH_2$	**V**	54.5	5.5	59.5	6.0	73.5	6.5
H-TGT ACG TCA CAA CTA-NH_2	**VI**	68.5		72.5		83.0	
H-TGT ACG TC**D** CAA CTA-NH_2	**VII**	71.5	3.0	75.0	2.5	>86	>3
H-GAGAGGAAAA-$LysNH_2$	**VIII**	67.0		66.0		69.0	
H-G**D**G**D**GG**DDDD**-$LysNH_2$	**IX**	>85	>3	>83	>3		
5′-dGTA GAT CAC T-3′		33.5		34.0		49.0	
5′-dGTA G**D**T CAC T-3′		36.0	2.5	36.5	2.5	52.5	3.5
5′-dGT**D**G**D**T C**D**C T-3′		44.0	3.5	42.5	3.0	61.0	4.0

[a]~4 μM in each strand.
[b]100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mmol EDTA, pH 7.0.
[c]DNA targets: 5′-AGT GAT CTA C-3′, 5′-GTA GAT CAC T-3′, 5′-TAG TTG TGA CGT ACA-3′.
[d]RNA targets: 5′-AGU GAU CUA C-3′, 5′-GUA GAU CAC U-3′, 5′-UAG UUG UGA CGU ACA-3′.
[e]PNA targets: H-AGT GAT CTA C-$LysNH_2$, H-GTA GAT CAC T-$LysNH_2$, H-TAG TTG TGA CGT ACA-$LysNH_2$.

Table 2. Decreases in thermal stabilities (ΔT_m) of duplexes between PNA oligomers containing diaminopurine (D) and RNA and PNA targets incorporating mismatches against the central diaminopurine[a,b]

Sequence		–ΔT_m (°C) for base pair mismatch								
		DNA[c]			RNA[d]			PNA[e]		
		A[f]	C	G	A	C	G	A	C	G
H-AGT G**A**T CTA C-$LysNH_2$	**I**	nd	nd	nd	11.5	11.0	11.5	13.0	17.0	13.0
H-AGT G**D**T CTA C $LysNH_2$	**II**	nd	nd	nd	16.0	13.5	20.5	20.0	14.0	24.0
H-TGT ACG TC**A** CAA CTA-NH_2	**VI**	15.0	12.0	13.0	11.0	8.0	9.0	nd	nd	nd
H-TGT ACG TC**D** CAA CTA-NH_2	**VII**	16.0	11.0	17.0	15.0	7.0	15.0	nd	nd	nd

[a]~4 μM in each strand.
[b] 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mmol EDTA, pH 7.0.
[c]DNA target: 5′-TAG TTG **X**GA CGT ACA-3′.
[d]RNA targets: 5′-GUA GA**X** CAC U-3′, 5′-UAG UUG **X**GA CGU ACA-3′.
[e]PNA targets: H-GTA GA**X** CAC T-$LysNH_2$.
[f]Base pair mismatch A/D:X, where X = A, C or G.
nd, not determined.

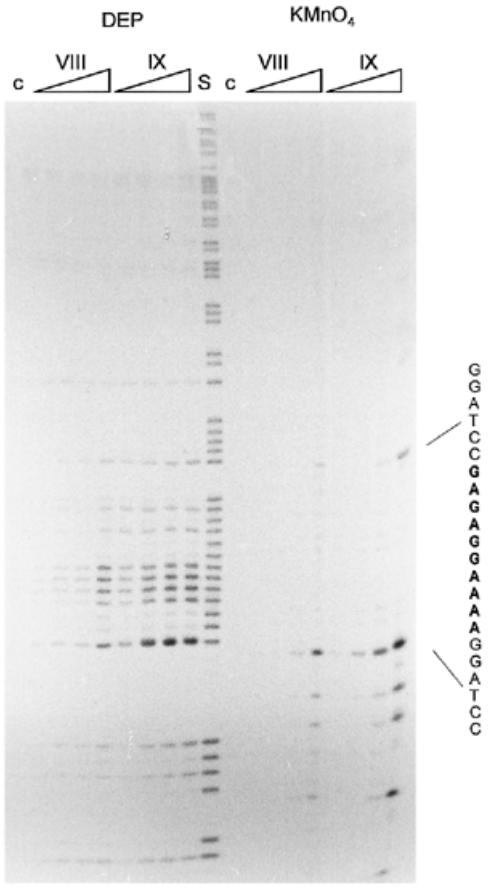


Figure 2. Chemical probing of strand displacement complexes formed by PNAs **VIII** and **IX**. Increasing concentrations of PNA were used (0.5, 1.5, 5 or 15 μM) and the complexes were probed with either diethylpyrocarbonate (DEP) or $KMnO_4$. Lanes 'c' are controls without PNA while lane 'S' is an A + G sequence reaction. A ^{32}P-end-labeled 247 bp double-stranded DNA fragment containing an antiparallel target for the PNAs was used.

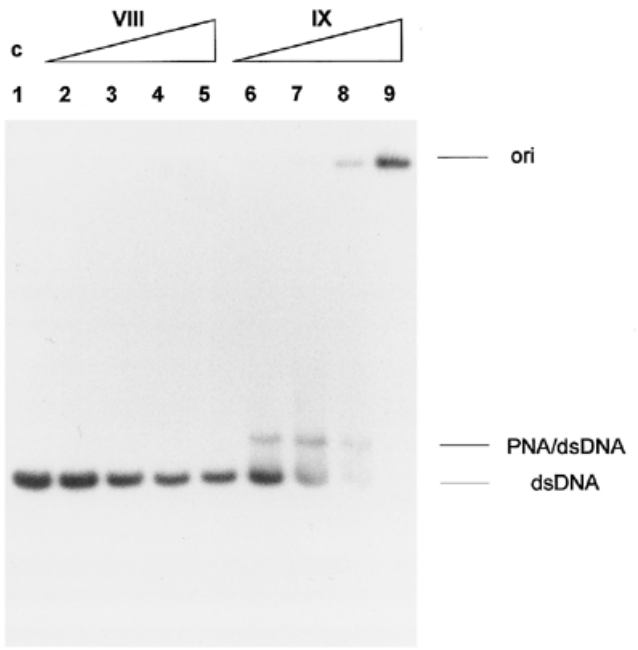


Figure 3. Electrophoretic mobility shift assay of binding of PNAs **VIII** and **IX** to a 247 bp double-stranded DNA fragment containing an antiparallel target for the PNAs. The following PNA concentrations (lanes 2–5 and 6–9) were used: 0.5, 1.5, 5 or 15 μM. Lane c is a control without PNA.

*2-Amino-6-(benzyloxycarbonylamino)purin-9-ylacetic acid (**4**).* Ethyl-2-amino-6-(benzyloxycarbonylamino)purin-9-ylacetate (**3**) (3.00 g, 8.1 mmol) was added to 2 M aqueous NaOH (30 ml). Dissolution occured after 1 h and the reaction was then cooled to 0°C and the product precipitated by acidifying to pH 2.5 with 2 M aqueous HCl. The solid produced was filtered, washed with water and dried yielding the title compound (**4**) (2.82 g, 98%) as a white solid. Mp > 260°C; IR (KBr): 3330, 3095, 1750, 1630, 1590, 1410/cm; ^{1}H NMR: δ 10.11 (s, 1H), 7.91 (s, 1H), 7.45–7.33 (m, 5H), 6.40 (s, 2H), 5.17 (s, 2H), 4.83 (s, 2H); ^{13}C NMR: δ 169.3, 160.0, 154.3, 149.9, 140.9, 140.9, 136.6, 128.4, 127.9, 127.8, 116.9, 66.0, 43.7; MS (FAB$^+$) *m/e* calculated for $C_{15}H_{15}N_6O_4$, 343.1155; found, 343.1158 $(M+H)^+$.

*Ethyl-N-(2-(t-butyloxycarbonylamino)ethyl)-N-(2-amino-6-(benzyloxycarbonylamino)purin-9-ylacetyl)glycinate (**5**).* A solution of 2-amino-6-(benzyloxycarbonylamino)purin-9-ylacetic acid (**4**) (2.00 g, 5.84 mmol), DhbtOH (1.05 g, 6.44 mmol) and DCC (1.33 g, 6.45 mmol) in dry DMF (25 ml) was stirred at room temperature for 1 h after which ethyl-*N*-(2-(t-butyloxycarbonylamino)ethyl)glycinate (1.50 g, 6.45 mmol) was added. The reaction was stirred for 16 h. DCU was removed by filtration and DMF removed *in vacuo*. Dichloromethane (50 ml) was added and the organic layer was washed with saturated $NaHCO_3$ (3×), saturated $KHSO_4$ (2×) and finally with brine. After drying ($MgSO_4$) and removal of solvent the white foam was crystallized from EtOH/H_2O to afford the title compound (**5**) (3.40 g, 74 %) as a white powder. ^{1}H NMR: δ 10.05 (s, 1H), 7.78 (s, 1H), 7.0 (m, 5H), 6.98 and 6.73 [m, major and minor rotamer (maj/min) respectively, 1H], 6.28 (br s, 2H), 5.17 (s, 2H), 5.07 and 4.91 [s (maj/min), 2H], 4.40 and 4.10 [s (maj/min), 2H], 4.28 and 4.12 [q, *J* = 7.1Hz, (min/maj), 2H], 1.38 and 1.37 [s (maj/min), 9H], 1.26 and 1.17 [q, *J* = 7.1 Hz (maj/min), 3H]; ^{13}C NMR: d 169.0, 167.0, 159.9, 155.8, 154.5, 152.3, 149.8, 141.2, 136.6, 128.4, 127.9, 127.8, 116.9, 78.2, 66.0, 60.6, 49.3, 47.1, 43.1, 38.4, 28.2, 14.1.

*N-(2-(t-Butyloxycarbonylamino)ethyl)-N-(2-amino-6-(benzyloxycarbonylamino)purin-9- ylacetyl)glycine (**6**).* A solution of ethyl-*N*-(2-(t-butyloxycarbonylamino)ethyl)-*N*-(2-amino-6-(benzyloxycarbonylamino)purin-9-ylacetyl)glycinate (**5**) (2.0 g, 3.59 mmol) in 2 M aqueous NaOH (20 ml) and THF (15 ml) was stirred for 1 h, after which most of the THF was removed *in vacuo* and the aqueous layer cooled to 0°C. The product was precipitated by adjusting the pH to 4 with 4 M HCl, yielding a solid which was washed extensively with water. Drying under high vacuum gave the title compound (**6**) (1.60 g, 82%) as a white solid. ^{1}H NMR: δ 7.91 (s, 1H), 7.53–7.40 (m, 5H), 7.05 (m, 1H), 6.46 (br s, 2H), 5.26 (s, 2H), 5.15 and 4.98 [s (maj/min), 2H], 4.39 and 4.07 [s (maj/min), 2H], 1.46 (s, 9H); ^{13}C NMR : δ 170.4, 166.7, 159.4, 155.8, 154.4, 152.6, 149.4, 141.4, 136.5, 128.4, 128.0, 127.8, 78.2, 66.2, 60.6, 49.24, 47.8, 43.3, 38.4, 28.2; MS (FAB$^+$) *m/e* calculated for $C_{24}H_{31}N_8O_7$, 543.2315; found, 543.2313 $(M+H)^+$.

RESULTS AND DISCUSSION

The PNA diaminopurine monomer was synthezied as shown in Scheme 1. Briefly, 2,6-diaminopurine was alkylated at N9 with ethyl bromoacetate followed by acylation at N6 with the triflate of Rapoport's reagent. Subsequently the ethyl ester was hydrolyzed with dilute sodium hydroxide and the resulting 2-amino-6-(benzyl-oxycarbonylamino)purin-9-ylacetic acid (**4**) was coupled to ethyl-*N*-(2-t-butyloxycarbonylaminoethyl)glycinate utilizing DCC and DhbtOH. The diaminopurine monomer (**6**) was finally obtained after hydrolysis with dilute sodium hydroxide.

PNA oligomers were synthesized by a modified Merrifield method as previously reported (13), except that capping was omitted subsequent to incorporation of the first diaminopurine moiety in the oligomer, as this could result in acetylation of the unprotected 2-amino group.

Four decameric (**II**, **III**, **V** and **IX**) and one pentadecameric (**VII**) PNA containing diaminopurine units were synthesized and the thermal stabilities of complexes between these PNAs and complementary DNA, RNA and PNA oligomers were measured (Table 1). It is consistently observed that substituting a diamino-purine for an adenine increases the thermal stability (ΔT_m) of the complexes by 4–6°C per substitution for the decamers and a little less for the pentadecamer. This increase (as compared with the decamers) appears slightly higher than that observed for the corresponding DNA oligomers (ΔT_m = 2.5–4°C).

Using oligomers **I**, **II**, **VI** and **VII** we also studied base recognition and discrimination by the diaminopurine (Table 2). Not surprisingly, diaminopurine, being 'the perfect three hydrogen bond complement of thymine', recognizes thymine and, in general (apart from cytosine), discrimination ($-\Delta T_m$) against mismatches opposite the diaminopurine in a DNA, RNA or PNA complement is somewhat higher with diaminopurine-containing PNAs than with normal adenine-containing PNAs. We also observed that discrimination against mismatches proximal to the diaminopurine was slightly increased as compared with discrimination by the normal adenine-containing PNA [C^8-X (A,C or T) in PNA **VII** versus **VI**] ($\Delta\Delta T_m$ = 1.5, 2.0 and 3.0°C for the C-A, C-C and C-T mismatches respectively).

Therefore, employment of diaminopurine in place of adenine in PNA yields oligomers that bind both more efficiently and most often with increased sequence specificity to complementary DNA, RNA or PNA oligomers.

We have recently shown that the homopurine decamer PNA **VIII** is able to bind to a double-stranded DNA target via strand invasion despite the fact that this PNA only forms a duplex, albeit of extraordinary high stability, with the complementary oligonucleotide (14). However, this strand displacement complex is much less stable than the corresponding triplex formed by a homopyrimidine PNA (e.g. H-TTTTCCTCTC-LysNH_2) and does not, for example, allow for analysis by gel shift assay.

As shown by the chemical probing experiments presented in Figure 2, both PNAs **VIII** and **IX** bind to the double-stranded DNA target by strand invasion: adenines within the target become sensitive to reaction with diethylpyrocarbonate, while hyper-reactivity of thymines to permanganate is induced proximal to the bound PNA. It is also seen from these results that the diaminopu-rine PNA **IX** is ~10-fold more potent than the standard PNA **VIII**. In fact, the strand displacement complex formed by PNA **IX** has sufficient stability to be analyzed by gel shift assay (Fig. 3), although higher concentrations of the PNA result in DNA aggregation, as evidenced by the non-migrating DNA at the top of the gel (Fig. 3, lanes 8 and 9).

Conclusion

The present results clearly demonstrate that it should be highly advantageous to substitute one or several adenines by diaminopurine in PNA oligomers for use as antigene or antisense therapeutic agents (8,16–18) and as diagnostic or molecular biology tools (19–23).

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