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Inhibition of PNA triplex formation by N^4-benzoylated cytosine

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ABSTRACT

The synthesis of *N*-((*N4*-(benzoyl)cytosine-1-yl)acetyl)-*N*-(2-Boc-aminoethyl)glycine (C^{Bz}) and the incorporation of this monomer into PNA oligomers are described. A single C^{Bz} residue within a 10mer homopyrimidine PNA is capable of switching the preferred binding mode from a parallel to an antiparallel orientation when targeting a deoxyribonucleotide sequence at neutral pH. The resulting complex has a thermal stability equal to that of the corresponding PNA–DNA duplex, indicative of a strong destabilization of Hoogsteen strand PNA binding due to steric interference by the benzoyl moieties. Accordingly, incorporation of the C^{Bz} residue into linked PNAs (bis-PNAs) results in greatly reduced thermal stability of the formed PNA:DNA complexes. Thus, incorporation of the C^{Bz} monomer could eliminate the stability bias of triplex-forming sequences in PNA used in hybridization arrays and combinatorial library formats. Furthermore, it is shown that the benzoyl moiety does not severely interfere with Watson–Crick hydrogen bonding, thereby presenting an interesting route for novel cytosine modifications.

INTRODUCTION

The pseudo-peptide DNA mimic PNA (peptide nucleic acid) may find widespread applications in molecular biology, medical diagnostics and therapy due to favorable hybridization properties, high chemical and biological stability and easy chemical synthesis of PNA (1–9). One of the unique properties of PNA is the ability of homopyrimidine PNAs to form PNA_2–nucleic acid triplexes of much higher thermal stability than the corresponding PNA–nucleic acid duplexes (2,10,11). This extraordinary stability of triplexes is essential for some of the unique properties of PNA, such as translation arrest (8,12) and strand displacement (1,3). Conversely, the large difference in stability between PNA_2–nucleic acid triplexes and duplexes could be a serious drawback for applications that require selective capture (e.g. hybridization array systems for diagnostics, DNA sequencing or gene expression analyses; 13), because the selection would be biased for sequences that permit triplex formation. Therefore PNA homopyrimidine oligomers that cannot form triplexes or form triplexes of reduced stability would be of interest. Since the formation of PNA_2–DNA complexes requires access to the major groove of a PNA–DNA duplex, an extended nucleobase blocking the major groove should destabilize binding of the third strand. Computer aided molecular model building indicated that a benzoyl substituent on the exocyclic 4-amino group of cytosine could serve this purpose without compromising Watson–Crick hydrogen bonding recognition. We describe here the synthesis of the C^{Bz} PNA monomer *N*-((*N*4-(benzoyl)cytosin-1-yl)acetyl)-*N*-(2-Boc-aminoethyl)glycine. We demonstrate that a mixed purine/pyrimidine decamer PNA containing one C^{Bz} monomer has close to retained the hybridization efficiency and discrimination of the parent PNA, whereas incorporation of a single C^{Bz} residue into a homopyrimidine PNA decamer changes the preferred binding orientation and greatly reduces the thermal stability of the complex to that expected for the corresponding PNA–DNA duplex.

MATERIALS AND METHODS

The nucleoside phosphoamidates were purchased from CruaChem (UK) and the DNA oligomers were assembled on a MilliGen/Biosearch 8700 DNA synthesizer. The A, C, G and T Boc-protected PNA monomers and *N*′-(2-Boc-aminoethyl)glycine were purchased from PerSeptive Biosystems (USA). All PNA oligomers were synthesized by a modified Merrifield procedure (5–7) and purified by RP-HPLC. The PNA oligomers were homogeneous by HPLC and showed the expected mass by FAB^+MS and/or MALDI-TOF MS (Kratos Maldi II).

Benzyl cytosin-1-yl acetate (1)

To cytosine (20 g, 0.18 mol) in DMF (400 ml) was added NaH (7.2 g, 0.18 mol, dispersed in oil 60%). The mixture was heated to 50°C and stirred for 2 h under nitrogen. After cooling to room temperature benzyl bromoacetate (29 ml, 1.1 equivalents) was added drop-wise. Following stirring overnight the dark suspension was filtered and the filtrate was washed with cold DMF and 0.2 M sodium bicarbonate. Crystallization from ethanol yielded 37 g (79%) of the title compound.

^{1}H-NMR (DMSO-d_6/TMS): δ = 4.56 (s, 2 H, CH_2CO), 5.24 (s, 2 H CH_2O), 5.77 (d, 1 H, H5), 7.20 (dd, 2 H, NH_2), 7.45 (m, 5 H, aromatics), 7.65 (d, 1 H, H6). ^{13}C-NMR (DMSO-d_6/TMS): δ = 93.6 (C-5); 127.9, 128.1, 128.4, 135.8 and 146.3 (aromatics C-2, C-4, C-6); 168.4 (CO); 168.7 (CO). MS (FAB) *m*/*z* 260 (M+H).

(N^4-(Benzoyl)cytosin-1-yl)acetic acid (2)

To a solution of **1** (10 g, 38 mmol) in pyridine (100 ml) was added benzoylchloride (6.6 g, 47 mmol). After stirring overnight at room temperature the solvent was evaporated under reduced pressure. The remains dissolved in 1 M KOH after stirring for 3 h.

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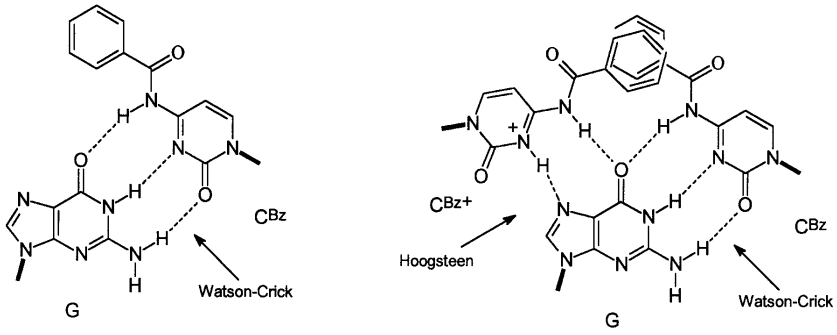


Figure 1. Schematic drawing of a G-C^{Bz} base pair and a C-G·C^{Bz} triplet indicating the steric clash with the benzoyl group.

Upon adjusting the pH to 2 with concentrated HCl the title compound precipitated. Yield 9.3 g (90%).

^{1}H-NMR (DMSO-d$_6$/TMS): δ = 4.59 (s, 2 H, C$\underline{H}_2$CO), 7.31 (d, 1 H, H6), 7.5–8.2 (7 H, aromatics, H5). MS (FAB) *m*/*z* 273 (M+H). mp 276°C (decomp.). ^{13}C-NMR (DMSO-d$_6$/TMS): δ = 50.7 (CH_2); 96.0 (C-5); 128.5, 133.0 and 133.3 (Bz); 150.8 (C-6); 155.2 (C-2); 163.6 (C-4); 167.5 (CO); 169.4 (CO).

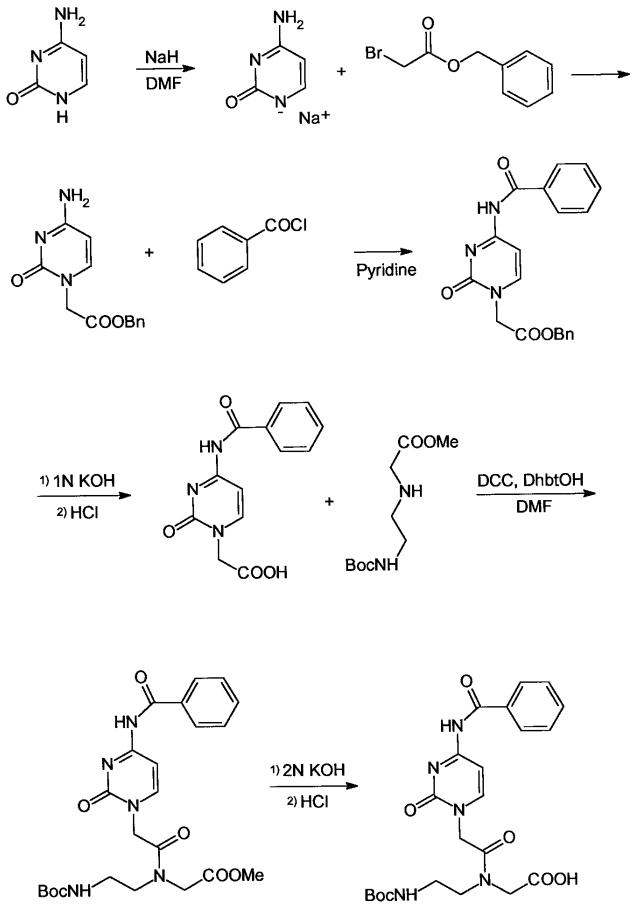
N-((*N*^{4}-(Benzoyl)cytosin-1-yl)acetyl)-*N*-(2-tBoc-aminoethyl) glycine (3)

Methyl *N*-(2-Boc-aminoethyl)glycinate (**7**) (4.8 g, 22 mmol), (*N*^{4}-(benzoyl)cytosin-1-yl)acetic acid (**2**) (2.4 g, 14.7 mmol), DCC (2.9 g, 14.9 mmol) and DhbtOH (3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine) (2.4 g, 14.7 mmol) was dissolved in DMF (50 ml) and stirred for 4 h at room temperature. Following addition of dichloromethane (100 ml) the solution was extracted with 0.2 M sodium bicarbonate (3 × 30 ml), 1 M sodium hydrogen sulfate (2 × 30 ml) and brine. The organic phase was dried over magnesium sulfate and evaporated to dryness under reduced pressure. The remains were dissolved in 2 M KOH and stirred for 1 h. Upon adjusting the pH to 2 with 1 M HCl the title compound precipitated. Crystallization from methanol:ethyl acetate:hexane (1:2:2) yielded 4.2 g (60%).

^{1}H-NMR (DMSO-d$_6$/TMS): δ = 1.45 and 1.47 (d, 9 H, Boc), 3.28–3.53 (m, 4 H, C$\underline{H}_2$), 4.08 and 4.31 (s, 2 H, C$\underline{H}_2$CO), 4.75 and 4.95 (s, 2 H, C$\underline{H}_2$CO), 6.83 and 7.03 (m, 1 H, BocN$\underline{H}$), 7.38 (m, 1 H, H5), 7.57–8.10 (m, 6 H, aromatics and H6). ^{13}C-NMR (DMSO-d$_6$/TMS): δ = 28.2 (Boc); 37.9 and 38.2 (NCH_2, minor and major rotamer); 47.0 and 47.8 (CH_2N, minor and major); 49.4 and 49.6 (CH_2CON, minor and major); 95.8 (C-5); 132.7 (C-4);151.0 (C-6); 163.3 (CON); 166.9 and 167.7 (CON, minor and major); 170.4 (COOH). MS (FAB) *m*/*z* 474 (M+H) (calculated 474). Calculated for $C_{22}H_{27}N_5O_7 \cdot 0.5H_2O$: C, 54.75; H, 5.84; N,14.52. Found: C, 54.87; H, 5.74; N, 14.53.

T_m measurements

Absorbance versus temperature was measured at 260 nm using a Gilford Response spectrophotometer. The heating rate was 0.5°C/step (~0.7°C/min) from 5 to 90°C. PNA oligomers were hybridized with complementary DNA (or PNA) oligomers in a buffer containing 100 mM NaCl, 10 mM Na phosphate and 0.1 mM EDTA, at pH 5, 7 or 9 as desired. 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.



Scheme 1. Synthesis of the C^{Bz} PNA monomer.

RESULTS AND DISCUSSION

The C^{Bz} monomer was synthesized as shown in Scheme 1. Cytosine was alkylated at N1 with benzyl bromoacetate followed by acylation at N4 with benzoylchloride. Subsequently the benzyl ester was hydrolyzed with diluted potassium hydroxide. The resulting (*N*^{4}-(benzoyl)cytosin-1-yl)acetic acid was coupled to methyl *N*-(2-Boc-aminoethyl)glycinate using DCC and DhbtOH. *N*-((*N*^{4}-(Benzoyl)cytosin-1-yl)acetyl)-*N*-(2-Boc-aminoethyl)-glycine (C^{Bz}) was finally isolated after hydrolysis with diluted potassium hydroxide.

Table 1. Thermal stability (T_m, °C)[a] for binding of PNA to single-stranded homopurine DNA oligomers

	5′-d(AAAAGGAGAG)-3′ (ODN1)	5′-d(GAGAGGAAAA)-3′ (ODN2)	pH
H-TTTTCCTCTC-LysNH_2 (PNA1)	>85.0	69.5	5
	58.5 (31.0)	40.5	7
	26.0	33.5	9
H-TTTTCC^{Bz}TCTC-LysNH_2 (PNA2)	56.0 (38.0)	54.0 (42.5)	5
	27.0 (20.0)	32.0 (29.0)	7
	n.d.[b]	31.0 (29.0)	9
H-TTTTCCTCTC-$(eg1)_3$-CTCTCCTTTT-LysNH_2 (PNA3)[c]	>90.0 (79.0) [>90]		5
	76.0 (67.0)	72.0 (64.0)	7
	42.0 (38.0)		9
H-TTTTCCTCTC-$(eg1)_3$-CTCTCC^{Bz}TTTT-LysNH_2 (PNA4)	>90.0 [71.5]		5
	66.0	57.0 (56.0)	7
	36.0		9
H-TTTTC^{Bz}CTCTC-$(eg1)_3$-CTCTCC^{Bz}TTTT-LysNH_2 (PNA5)	75 [56.8]		5
	55.0	49.0 (49.0)	7
	32.0		9
H-TTTTCC^{Bz}TCTC-$(eg1)_3$-CTCTCC^{Bz}TTTT-LysNH_2 (PNA6)	79		5
	54.0	49.0 (49.0)	7
	32.0		9
H-TTTTCCTC^{Bz}TC-$(eg1)_3$-CTCTCC^{Bz}TTTT-LysNH_2 (PNA7)	87		5
	58.0	49.0 (49.0)	7
	33.0		9
H-TTTTCC^{Bz}TC^{Bz}TC-LysNH_2 (PNA12)	28.0	33.0	7
H-TTTTC^{Bz}C^{Bz}TCTC-LysNH_2 (PNA13)	26.0	32.5	7

Absorbance versus temperature curves were measured at 260 nm in 100 mM NaCl, 10 mM Na phosphate, 0.1 mM EDTA. Heating rate, 0.5°/min from 5 to 90°C. The T_m values in parentheses were obtained by cooling from 90 to 10°C while measuring the absorbance at 260 nm. The T_m values in brackets were obtained in 20% formamide buffer, 100 mM NaCl, 10 mM Na phosphate, 0.1 mM EDTA.
n.d., not detected.
[a]If not otherwise stated, all T_m values are accurate to within 1°C.
[b]The melting curve did not give a well-defined T_m (estimated 15–25°C).
[c]'eg1' denotes the 'ethyleneglycol' linker 8-amino-3,6-dioxaoctanoic acid.

In order to study the effect of a C^{Bz} residue on the hybridization properties of a homopyrimidine PNA, we synthesized the two PNAs H-TTTTCCTCTC-LysNH_2 (PNA1) and H-TTTTCC^{Bz}TCTC-LysNH_2 (PNA2). These PNAs were hybridized to a complementary oligonucleotide in the parallel (amino end of the PNA facing the 5′-end of the oligonucleotide) (ODN1) or the antiparallel (ODN2) orientation and the thermal stability (T_m) of the resulting complexes was determined at pH 5, 7 and 9 (Table 1). The T_m of the complexes between the unmodified PNA1 and the complementary ODN1 or ODN2 showed a large pH dependence compatible with PNA_2–DNA triplex formation requiring cytosine protonization. Furthermore, the parallel complex (ODN1) has higher stability than the antiparallel complex at pH 5 and 7, but not at pH 9. An important feature distinguishing PNA–DNA duplexes from PNA_2–DNA triplexes under thermal denaturation conditions is a small hysteresis of the transition for duplexes (<2°C), whereas triplexes show very large hysteresis (typically in the range 20–30°C) (10). At pH 7 a large hysteresis (~27°C) was observed for the PNA1–ODN1 complex consistent with triplex formation.

PNA2 containing one C^{Bz} residue in the middle was likewise hybridized to the complementary parallel and antiparallel DNA oligomers. At pH 5 the parallel orientation is slightly preferred over the antiparallel orientation, but with a T_m of 56°C that is ~30°C lower than that of the unmodified PNA1–ODN1 complex. Attempts to determine the stoichiometry (at 20°C) of the complexes by titration (Job plot) did not give a clear minimum, but rather a flat curve between molar ratios 0.5–0.67. However, both complexes show higher stability at pH 5 than at pH 7 and a large hysteresis at pH 5, suggesting a 2:1 stochiometry under acidic conditions. These observations imply that the benzoyl groups do indeed interfere with, but do not prevent (at pH 5), triplex formation. The effect of the C^{Bz} unit is even greater at pH 7, where the antiparallel complex (ODN2) has the highest stability. Furthermore, this complex exhibits only a small hysteresis and the T_m shows no pH dependence between pH 7 and 9, indicating that the thermal transition is due to duplex dissociation.

It should be noted that the antiparallel DNA complexes with PNA1 and PNA2 show comparable thermal stability at pH 9

(assigned to the duplex), thus indicating that the C^{Bz} residue does not severely interfere with antiparallel Watson–Crick base pairing in the PNA–DNA duplex. In order to substantiate this conclusion we hybridized the PNA oligomers H-AGTCAC^{Bz}CTAC-LysNH_2 (PNA14) and H-AGTCACCTAC-LysNH_2 (PNA15) (Table 2) to their complementary antiparallel oligonucleotide target (ODN3). Both PNAs form stable duplexes with only slightly reduced thermal stability of the C^{Bz}-containing PNA. The stoichiometry of these complexes were confirmed by Job plots (data not shown) as 1:1 in both cases. Therefore, the benzoyl group in the major grove of the PNA–DNA helix (Fig. 1) does not severely interfere with the Watson–Crick base pairing. Accordingly, mismatch substitutions in the complementary oligonucleotide (ODN4–6) opposite the C^{Bz} residue resulted in substantial and comparable decreases in T_m for the duplexes of both PNA14 and PNA15. However, because of the significant thermal transition ('self-melt') around 40°C exhibited by both PNAs, it was not possible to determine accurately the T_m of the mismatched PNA–DNA duplexes. Therefore, analogous experiments were performed with PNA–PNA duplexes, which have significantly higher thermal stabilities (Table 1), and these results clearly demonstrate that the C^{Bz} nucleobase discriminates essentially as efficiently as cytosine for binding to guanine.

In order to examine whether the C^{Bz} residue was needed in both strands of the PNA to prevent triplex formation, we synthesized a number of bis-PNAs containing no, one or two C^{Bz} residues at varying positions (PNA3–7, Table 1). In these bis-PNAs the two 'PNA halfs' are antiparallel to each other and therefore PNA_2–DNA triplexes with the preferred antiparallel Watson–Crick and parallel Hoogsteen configuration may be formed, in contrast to triplexes with 'mono PNAs', in which both PNA strands naturally have identical orientation. The T_m of complexes between these bis-PNAs and the complementary oligonucleotide ODN1 were determined at pH 5, 7 and 9. The thermal stabilities of all the complexes display a strong pH dependency and the results at pH 5 (in 20% formamide buffer) show that a single benzoyl group (PNA4) decreases the thermal stability of the triplex by >18.5°C, whereas two C^{Bz} residues (PNA5), one in the Watson–Crick strand and one in the Hoogsteen strand, additionally reduces the stability of the complex by 15°C. It should be noticed that with increasing distance between the two benzoyl groups (PNA5–PNA7) the destabilizing effect of the benzoyl groups is reduced, suggesting that part of the destabilization of the triplex is due to steric interference between benzoyl moieties of the C^{Bz} units.

From the above results with PNA3–PNA7 we could not unambiguously assign the interfering effect of the benzoyl group to the Watson–Crick or the Hoogsteen strand, because binding could take place either with the presumably preferred antiparallel Watson–Crick/parallel Hoogsteen configuration or with the presumably less preferred opposite orientation. Based on sequence symmetry arguments, PNA3 should bind equally well to ODN1 and ODN2, but as observed previously (11), a small difference (ΔT_m = 4°C) ascribed to the asymmetry caused by the linker (11) is observed. PNA4 shows somewhat increased discrimination between ODN1 and ODN2 (ΔT_m = 9°C) and this could be taken as evidence that having the C^{Bz} unit in the Hoogsteen strand (ODN1) is more detrimental than having it in the Watson–Crick strand (ODN2). Incorporating pseudoisocytosine (J) into the parallel strand in place of cytosine is a way to further direct this to form the Hoogsteen strand of the complex at neutral or basic pH (11), because this nucleobase functions as a permanently protonated cytosine and can therefore form a two hydrogen bonded Hoogsteen pair with guanine independent of pH. The results (Table 3) showed a small effect (2–5°C) of the C^{Bz} substitution when incorporated into the Watson–Crick strand (PNA8 and PNA9), whereas the decrease in T_m was >6°C at pH 5 and 22°C at pH 7 and 9 upon incorporating C^{Bz} into the Hoogsteen strand (PNA10 and PNA11). Thus, having the C^{Bz} substitution in the Hoogsteen strand gives the larger destabilization of the triplex. However, this could, at least in part, also be due to a considerably lowered pK_a for the protonation of N3 of C^{Bz} and not solely to a steric effect.

Finally, by analogy with previous reports using extended pyrimidines (14), we speculated if adjacent C^{Bz} nucleobases could stabilize the complex by increased stacking or hydrophobic interactions. Thus, H-TTT TCC^{Bz}TC^{Bz}TC-LysNH_2 (PNA12) and H-TTTTC^{Bz}C^{Bz}TCTC-LysNH_2 (PNA13) were synthesized. The hybrids of DNA2 and the two PNAs showed virtually no difference in thermal stability and positive stacking interactions between two adjacent C^{Bz} moieties does not seem to occur.

Table 2. Thermal stablity (T_m, °C) mismatch analysis of PNA–DNA and PNA–PNA duplexes

	H-AGTGACCTAC-LysNH_2 (PNA14)	H-AGTGAC^{Bz}CTAC-LysNH_2 (PNA15)
5′-d(GTAGGTCACT)-3′ (ODN3)	54.5	52
5′-d(GTAG**A**TCACT)-3′ (ODN4)	≤45[a]	≤43[a]
5′-d(GTAG**C**TCACT)-3′ (ODN5)	≤45[a]	≤43[a]
5′-d(GTAG**T**TCACT)-3′ (ODN6)	≤44[a]	≤45[a]
H-GTAGGTCACT-NH_2	73.5	70.5
H-GTAG**A**TCACT-NH_2	50.0	52.0
H-GTAG**C**TCACT-NH_2	51.0	50.0
H-GTAG**T**TCACT-NH_2	50.5	50.0

Melting temperatures (T_m, °C) for binding of PNA in duplex mode to single-stranded DNA oligomer. Absorbance versus temperature curves were measured at 260 nm in 100 mM NaCl, pH 7, 10 mM Na phosphate, 0.1 mM EDTA. Heating rate, ~0.7°/min from 5 to 90°C.
[a]Due to a thermal transition around 40°C of the PNA itself this value is not accurately determined.

Table 3. Thermal stability (T_m, °C) of bis-PNA complexes

	5′-d(AAAAGGAGAG)-3′ (ODN1)	pH
H-TTTTJJTJTJ-(eg1)$_3$-CTCTCCTTTT-LysNH$_2$ (PNA8)	>90.0	5
	89	7
	80	9
H-TTTTJJTJTJ-(eg1)$_3$-CTCTCC^{BZ}TTTT-LysNH$_2$ (PNA9)	88.0	5
	84.0	7
	76.0	9
H-TTTTCJTJTJ-(eg1)$_3$-CTCTCCTTTT-LysNH$_2$ (PNA10)	>90	5
	85.0	7
	68.0	9
H-TTTTC^{BZ}JTJTJ-(eg1)^3-CTCTCCTTTT-LysNH^2 (PNA11)	84.0	5
	63.0	7
	46.0	9

Absorbance versus temperature curves were measured at 260 nm in 100 mM NaCl, 10 mM Na phosphate, 0.1 mM EDTA. J is the C^+ mimic pseudoisocytosine (11).

CONCLUSIONS

In conclusion, we have demonstrated that incorporation of a single C^{Bz} residue into a homopyrimidine mono PNA oligomer will change the preferred binding mode from a parallel 2:1 (PNA–DNA) complex to an antiparallel complex at neutral conditions. Only a slight decrease in the T_m was observed when the C^{Bz} residue was incorporated into a mixed sequence, whereas a single C^{Bz} residue was capable of reducing the thermal stability at neutral pH of homopyrimidine PNA_2–DNA triplexes to the level of the corresponding PNA–DNA duplexes. Thus, incorporation of the C^{Bz} residue into PNA may in general eliminate the stability bias of homopyrimidine sequences, which will be of importance for leveling out complex stabilities in hybridization-based selection systems. Furthermore, since the presence of the benzoyl group does not severely interfere with Watson–Crick recognition, this modification presents a novel route to chemically modified cytosines. This modification is, however, less accessible in phosphodiester oligonucleotides due to the ammonia deprotection step, which will effectively remove the benzoyl group. In fact, amides are versatile protecting groups in DNA synthesis.

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