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Insertion of an internal dipeptide into PNA oligomers: Thermal melting studies and further functionalization

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Abstract—The solid phase synthesis of PNA oligomers with the internal dipeptide Gly-Phe is presented and the interaction with complementary DNA investigated. UV absorbance melting experiments with different but complementary DNA sequences show that stable PNA•DNA duplexes are only obtained when there is no DNA base opposite the dipeptide unit. Instead, the dipeptide spacer forms a loop-like structure within the duplex. Further functionalization with N-heterocyclic ligands is described. *p*-Nitrophenylalanine is introduced in place of Phe during solid phase synthesis and subsequently reduced to *p*-amino-phenylalanine. Reaction with activated acids provides the ligand conjugates in high yield and purity. This strategy opens a universal route to a large number of internal substitutions in PNA chemistry.

Peptide nucleic acids (PNAs) are DNA analogs where the sugar-phosphate backbone is substituted by a pseudopeptide skeleton composed of *N*-(2-aminoethyl)-glycine units (Fig. 1).[1] PNA possesses a remarkable chemical stability and is neither degraded by nucleases nor proteases. In addition, PNA shows high specificity in binding DNA and RNA. These properties allow PNA to act as an antigene and antisense agent, respectively.

In recent literature, metal derivatives of PNA have received increased attention.[2] One notable example is the development of artificial nucleases[3] and proteases,[4] where PNA serves as the recognition domain and a metal complex as the cleavage domain. A metal complex can be introduced either at the N-terminal end of the oligomer[3–8] or at an internal site within the PNA sequence. Maiorana and co-workers[9] substituted the C2 atom of the PNA backbone and retained all nucleobases, while

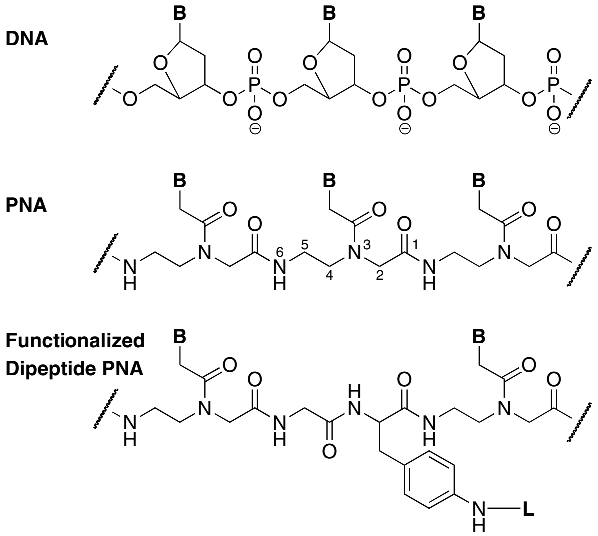

Figure 1. Comparison of DNA, PNA and functionalized dipeptide PNA (**B**, nucleobase; **L**, ligand).

Abbreviations: Bhoc, benzhydryloxycarbonyl; Boc, *tert*-butyloxycarbonyl; bpa, *N*-(4-carboxymethyl)benzyl-*N*,*N*-bis(2-picolyl)amine **6-OH**; DCM, dichloromethane; DIPEA, diisopropyl-ethylamine; DMF, dimethylformamide; Fmoc, fluorenyl-9-methoxycarbonyl; HATU, 2-(1*H*-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; PNA, peptide nucleic acid; SPPS, solid phase peptide synthesis; Su, succinimidyl; tpy, 4-[4′-oxa-(2,2′:6′,2″-terpyridinyl)]hexanoic acid **5-OH**; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

Keywords: Artificial nucleases; Bis(2-picolyl) amine; Peptide nucleic acid (PNA); Solid phase peptide synthesis; Terpyridine.

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Balasubramanian and co-workers,[10] Achim and co-workers,[11] and Williams and co-workers[12] reported on PNA oligomers where a nucleobase is exchanged with a nitrogen ligand capable of metal coordination. From a synthetic point of view, a major drawback of the published internal PNA modifications is that these monomers often have to be prepared in solution by a multistep procedure.

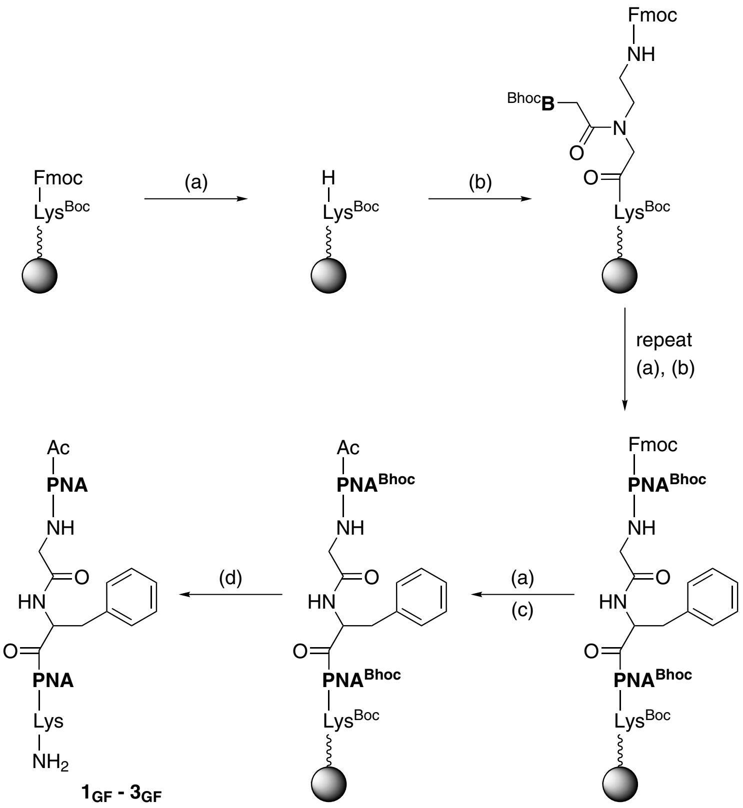
Here, we present a novel approach to functionalize PNA oligomers capable of metal coordination. The working hypothesis is that six atoms are added to the oligomer backbone using either one PNA monomer or one dipeptide.[13,14] In addition, both PNA and peptide chemistry are synthetically based on amide bond formation, performed by solid phase peptide synthesis (SPPS). Therefore, one PNA monomer can be substituted with one dipeptide (Fig. 1). We chose Gly-Phe as the dipeptide sequence, and we are aiming to functionalize the phenyl side chain group of Phe by substitution with a nitrogen ligand. If this substitution is performed on the resin during the course of SPPS, a universal strategy is obtained that opens up an avenue for a vast variety of substituents and saves the time-consuming monomer synthesis in solution.

Oligomers $\mathbf{1}_{\mathbf{O}}$–$\mathbf{3}_{\mathbf{O}}$, $\mathbf{1}_{\mathbf{C}}$–$\mathbf{3}_{\mathbf{C}}$ and $\mathbf{1}_{\mathbf{GF}}$–$\mathbf{3}_{\mathbf{GF}}$ were composed of two PNA chains (4, 5 or 6mer PNA each), without a central C, with A central C or with an internal Gly-Phe dipeptide, respectively (see Table 1 for exact sequences). They were synthesized on TentaGel R Fmoc-Lys(Boc)-RAM resin using Fmoc-/Bhoc-protected PNA monomers and Fmoc-protected amino acids (see Scheme 1, for the synthesis of $\mathbf{1}_{\mathbf{GF}}$–$\mathbf{3}_{\mathbf{GF}}$).[5a,15] The C-terminal lysine residue is introduced in order to enhance solubility. One synthetic cycle was composed of deprotection of the N-terminus (20% piperidine in DMF) and subsequent coupling of the respective PNA or amino acid monomer, using HATU/DIPEA in DMF. After each coupling step,

Table 1. Sequences and analytical data of PNAs **1**–**3** prepared in this study

PNA	Sequence	M_{calcd}	M_{found}[a]
$\mathbf{1}_{\mathbf{O}}$	Ac-tacc-tgtt-Lys-NH_2	2321.0	2321.7
$\mathbf{1}_{\mathbf{C}}$	Ac-tacc-c-tgtt-Lys-NH_2	2572.1	2573.1
$\mathbf{1}_{\mathbf{GF}}$	Ac-tacc-GlyPhe-tgtt-Lys-NH_2	2525.0	2526.7
$\mathbf{1}_{\mathbf{tpy}}$	Ac-tacc-GlyPhe(NH-**5**)-tgtt-Lys-NH_2	2885.2	2886.2
$\mathbf{1}_{\mathbf{bpa}}$	Ac-tacc-GlyPhe(NH-**6**)-tgtt-Lys-NH_2	2855.2	2855.1
$\mathbf{2}_{\mathbf{O}}$	Ac-ttacc-tgtta-Lys-NH_2	2862.2	2863.1
$\mathbf{2}_{\mathbf{C}}$	Ac-ttacc-c-tgtta-Lys-NH_2	3113.3	3115.1
$\mathbf{2}_{\mathbf{GF}}$	Ac-ttacc-GlyPhe-tgtta-Lys-NH_2	3066.3	3067.6
$\mathbf{2}_{\mathbf{tpy}}$	Ac-ttacc-GlyPhe(NH-**5**)-tgtta-Lys-NH_2	3426.4	3428.6
$\mathbf{2}_{\mathbf{bpa}}$	Ac-ttacc-GlyPhe(NH-**6**)-tgtta-Lys-NH_2	3967.6	3968.2
$\mathbf{3}_{\mathbf{O}}$	Ac-attacc-tgttat-Lys-NH_2	3404.4	3404.5
$\mathbf{3}_{\mathbf{C}}$	Ac-attacc-c-tgttat-Lys-NH_2	3655.0	3654.9
$\mathbf{3}_{\mathbf{GF}}$	Ac-attacc-GlyPhe-tgttat-Lys-NH_2	3608.5	3608.5
$\mathbf{3}_{\mathbf{tpy}}$	Ac-attacc-GlyPhe(NH-**5**)-tgttat-Lys-NH_2	3396.4	3397.8

[a] Determined by MALDI-TOF MS.



Scheme 1. Synthesis of unsubstituted dipeptide PNAs $\mathbf{1}_{\mathbf{GF}}$–$\mathbf{3}_{\mathbf{GF}}$. (a) Fmoc-deprotection: 20% piperidine/DMF (10 min); (b) coupling: monomer/HATU/DIPEA (1 min)/DMF (20 min); (c) acetylation: 5% Ac_2O/6% DIPEA/DMF (3 min); final cleavage: TFA/H_2O/TIS = 95:2.5:2.5 (3 h). Monomers: Fmoc-$\mathbf{B}^{\mathrm{Bhoc}}$-OH, Fmoc-Gly-OH or Fmoc-Phe-OH; **B** = a, c, g, t.

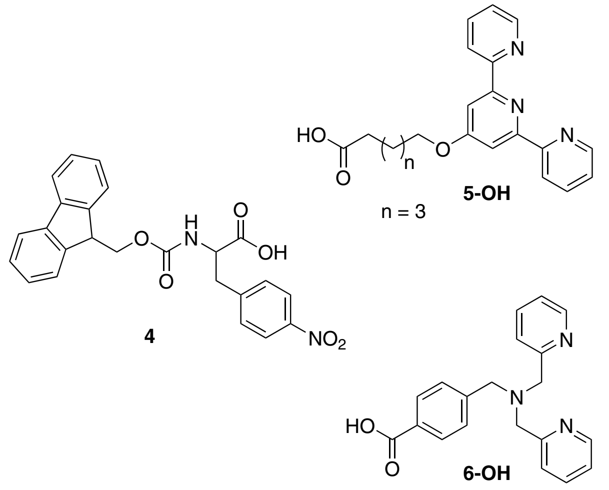
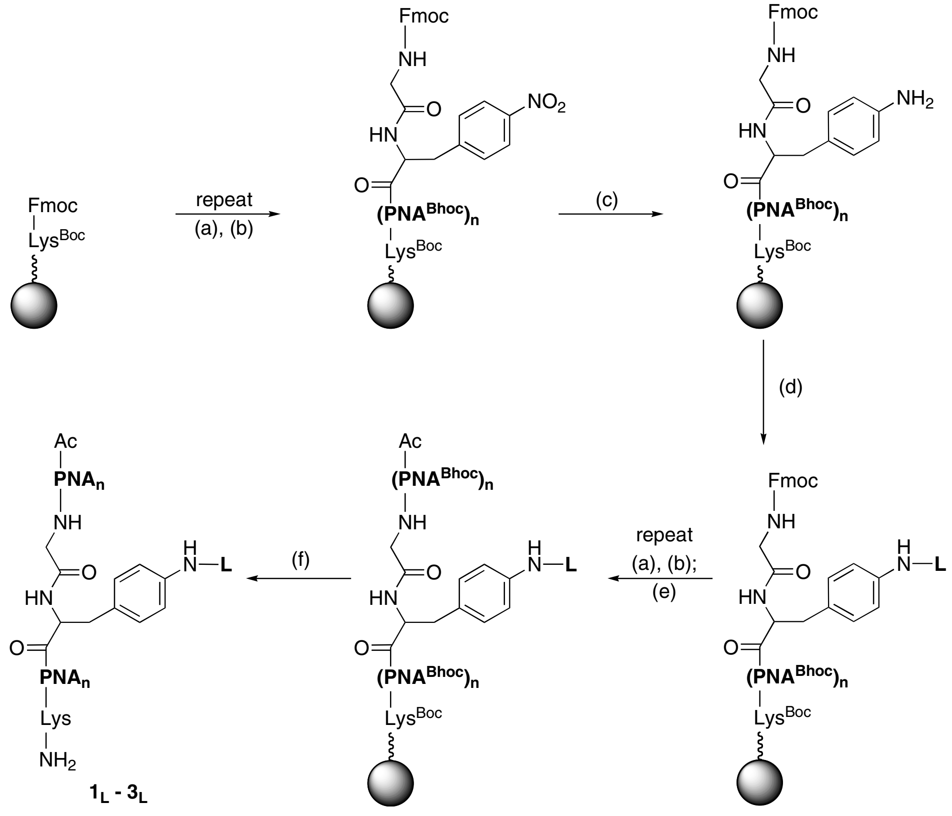


Figure 2. Monomer Fmoc-Phe(p-NO_2)-OH **4**, ligands **5-OH** (tpy) and **6-OH** (bpa).

a Kaiser test on free amine groups was performed. If unreacted amine groups were present, they were acetylated with 5% acetic anhydride and 6% DIPEA in DMF. After the last coupling step, the N-terminus was deprotected and acetylated, followed by cleavage of the final oligomer from the resin (95% TFA, 2.5% H_2O, 2.5% TIS) and precipitation with cold ether. Oligomers **1**–**3** were purified by RP-HPLC,[16] lyophilized and characterized by MALDI-TOF[17] (Table 1).

The Fmoc-Phe(p-NO_2)-OH amino acid **4** was used as a key precursor for the internally functionalized PNA oligomers (Fig. 2). It was prepared by nitration of phenylalanine with HNO_3/H_2SO_4, followed by Fmoc-protection of the amine group. Acid-functionalized terpyridine (tpy) **5-OH** and bis(2-picolyl) amine (bpa) **6-OH** served as ligands (Fig. 2). Ligand **5-OH** was synthesized by nucleophilic substitution of the corresponding chloride with ε-caprolactone using one equivalent of H_2O and an excess of KOH in dry DMSO.[18] Ligand **6-OH** was prepared starting from commercially available, unsubstituted bis(2-picolyl) amine and methyl 4-(bromomethyl)benzoate.[15,19]

For functionalization, the same PNA sequences as in $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$ were chosen, but the p-NO_2-Phe derivative **4** was used instead of the unsubstituted Phe. The sequences were assembled using the procedure described above; the nitro group was inert under these conditions (Scheme 2). Reduction of the nitro group was performed using 0.9 M $SnCl_2 \cdot 2H_2O$ in DMF on the resin.[20] Best results were obtained if the reduction was carried out twice after Gly had been added to the growing oligomer chain on the resin. Then, the ligands **5-OH** or **6-OH** were coupled to the p-amino function via their carboxylic group under standard conditions for amide bond



Scheme 2. Synthesis of substituted dipeptide PNAs $\mathbf{1_L}$–$\mathbf{3_L}$: internal coupling strategy of a ligand **L**. (a) Fmoc-deprotection: 20% piperidine/DMF (10 min); (b) coupling: monomer/HATU/DIPEA (1 min)/DMF (20 min); (c) reduction: 0.9 M $SnCl_2 \cdot 2H_2O$/DMF (16 h, 2×); (d) coupling: ligand **L-OH**/HATU/DIPEA (15 min)/DMF (16 h); (e) acetylation: 5% Ac_2O/6% DIPEA/DMF (3 min); (f) final cleavage: TFA/H_2O/TIS = 95:2.5:2.5 (3 h). Monomers: Fmoc-$\mathbf{B}^{Bhoc}$-OH, Fmoc-Gly-OH or Fmoc-Phe(p-NO_2)-OH; **B** = a, c, g, t; **L** = **5**, **6**.

formation (HATU/DIPEA). Finally, oligomer synthesis, isolation and purification were continued as described for $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$. The analytical data for oligomers $\mathbf{1_L}$–$\mathbf{3_L}$ (**L** = tpy, bpa) are also listed in Table 1.

UV melting experiments were performed in order to investigate the influence of dipeptide substitution in PNA oligomers on their DNA binding properties. To this end, PNA oligomers $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$ were hybridized to two different DNA oligomers. First, a DNA 18mer **7** was used. It has a sequence complementary to $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$ (bold) with a G monomer (underlined) opposite to the Gly-Phe dipeptide position: 3′-**TAATGGGACAAT** AGGGAT-5′. As expected, DNA **7** binds strongly and cleanly to the complementary PNAs $\mathbf{1_C}$–$\mathbf{3_C}$. However, only a linear increase of absorption rather than a sigmoidal melting curve was obtained even for the **3·7** duplex with the longest PNA sequence. The same result is observed for the Gly-Phe dipeptide PNAs ($\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$) (Fig. 3, hatched line for $\mathbf{3_{GF}}$**·7**). This result indicates that the hybridization is critically interrupted by Gly-Phe. Therefore, melting studies with $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$ and DNA **8** were conducted. DNA 17mer **8** has the same sequence as **7**, except that the **G** monomer is omitted: 3′-**TAATGGACAATA**GGGAT-5′. These experiments give a clear sigmoidal melting profile, see full line in Figure 3. The matching PNAs $\mathbf{1_{GF}}$–$\mathbf{3_{GF}}$ show the expected increase in stability with increasing PNA length and in all cases, insertion of the Gly-Phe dipeptide destabilizes the duplex by about 13–14 °C. As control experiments, the PNA oligomers were hybridized with DNA 'halves', that is, truncated sequences that match only the left or the right half of the PNA from the dipeptide unit on. In no case, a substantial DNA·PNA binding was ever observed. All T_m data are collected in Table 2. They suggest an extended PNA·DNA hybridization with the dipeptide forming a bulge, as known in single stranded RNA chemistry, Figure 4.

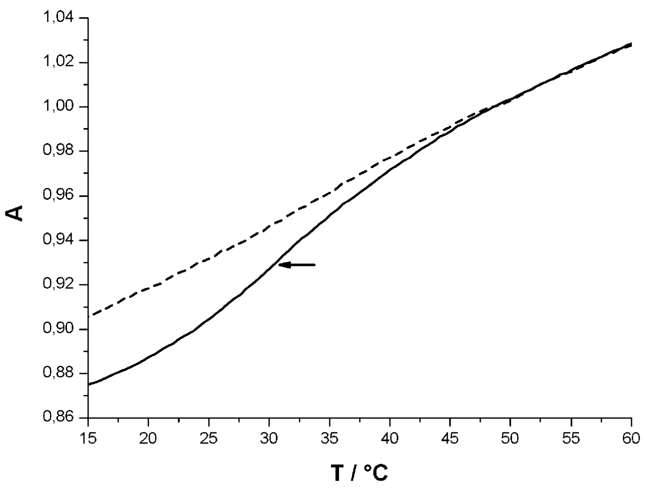


Figure 3. UV melting curves of $\mathbf{3_{GF}}$**·7** (hatched line) and $\mathbf{3_{GF}}$**·8** (full line). The melting temperature (T_m) is indicated with an arrow. The absolute absorbance of the $\mathbf{3_{GF}}$**·7** curve was adjusted to make the two curves overlap at higher temperatures solely to simplify visual comparison of the curves.

Table 2. UV–T_m data for PNAs **1**–**3** with two different DNA oligomers[a]

PNA	T_m/°C PNA·**7** (+G)	T_m/°C PNA·**8** (−G)
$\mathbf{1_O}$	n.o.[b]	25.1 ± 0.02
$\mathbf{1_C}$	41.8 ± 0.5	n.o.[b]
$\mathbf{1_{GF}}$	n.o.[b]	11.0 ± 0.1
$\mathbf{2_O}$	29.4 ± 1.6	35.3 ± 0.1
$\mathbf{2_C}$	47.4 ± 0.3	n.o.[b]
$\mathbf{2_{GF}}$	n.o.[b]	21.5 ± 0.1
$\mathbf{3_O}$	35.0 ± 0.7	43.2 ± 0.2
$\mathbf{3_C}$	52.1 ± 0.2	n.o.[b]
$\mathbf{3_{GF}}$	n.o.[b]	30.7 ± 0.2

[a] Melting temperatures T_m were determined by UV spectroscopy with DNA **7** (3′-**TAATGGGACAATA**GGGAT-5′) or DNA **8** (3′-**TAAT GGACAATA**GGGAT-5′) at 3 μM PNA and DNA concentration, phosphate buffered solution at pH 7 with a NaCl concentration of 100 mM. After pre-equilibration, the A_{260} was recorded against temperature (0.5 °C/min, 5–90 °C). The T_m values were determined as the maxima of the first derivative plots of A_{260} versus T, and are an average of four separate experiments ± standard deviation.

[b] n.o., no sigmoidal melting profile observed.

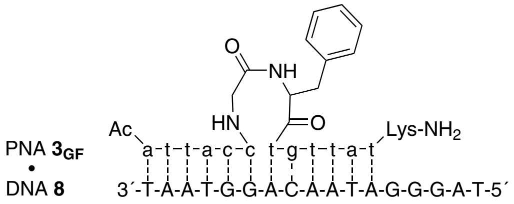


Figure 4. Hybridization of the $\mathbf{3_{GF}}$**·8** duplex with the Gly-Phe dipeptide bulge. Capital and small letters denote DNA and PNA monomers, respectively.

In conclusion, we describe the solid phase synthesis of PNA oligomers, where an internal PNA monomer is substituted by the Gly-Phe(p-NO_2) dipeptide. The dipeptide unit can be further functionalized. The key step is the reduction of the p-nitro group to a p-amino group on the resin by Sn(II) chloride. This strategy opens up a large number of possible substitutions by reaction of the amino group with activated acids, which is exemplified by coupling of the chelating ligands **5** and **6** in this work. Lewis and co-workers used a lysine–DOTA conjugate within a PNA oligomer for radiometal labeling.[13] We have recently reported the use of the Pd-catalyzed Sonogashira coupling using p-iodo-Phe for the synthesis of organometallic peptide bioconjugates.[21] The use of p-NO_2-Phe is more versatile in that it is orthogonally 'deprotected' by reduction to an amino group, and any carboxylic acid can subsequently be used for peptide derivatization. On the other hand, it requires an additional step in the reduction of the nitro group to an amino group. As shown herein, this step can be performed on the resin in high yield.

In order to establish whether dipeptide substitution impairs DNA binding of the peptide-substituted PNA, UV–T_m experiments with various complementary DNAs were performed. Interaction with complementary DNA is only possible when there is no

DNA nucleotide opposite the dipeptide unit. This result relates well to the work by Seitz and co-workers, who used a DNA matrix for the catalytic amplification of a PNA–dipeptide–PNA oligomer.[14] As expected, melting temperatures increase with increasing length of the PNA strands flanking the central dipeptide.

Unfortunately, the tpy and bpa functionalized PNA oligomers prepared in this work were only soluble at low pH. Therefore, no T_m experiments could be performed on these compounds. Further substitution of PNA oligomers with enhanced solubility is presently under investigation in our laboratory.

Acknowledgments

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