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Peptide Nucleic Acids with a Flexible Secondary Amine in the Backbone Maintain Oligonucleotide Binding Affinity

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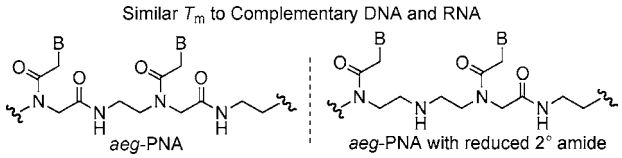
ABSTRACT



Replacing a secondary amide in a peptide nucleic acid backbone with a more flexible secondary amine affords an oligomer that surprisingly maintains the same binding affinity to complementary oligonucleotides as the unmodified polyamide oligomer.

In this communication, we report that a secondary amide in a peptide nucleic acid backbone can be replaced with a more flexible secondary amine without loss in binding affinity to complementary oligonucleotides. Peptide nucleic acids (PNAs) are a unique class of oligonucleotide mimics in which the sugar phosphate backbone is replaced with a polyamide backbone composed of *N*-(2-aminoethyl)glycine (*aeg*) units (Figure 1, A).[1] While *aeg*PNAs are used in several areas of genomic diagnostics, improvements in the properties of these oligomers would greatly extend their application to other areas of biomedical and biochemical research. For example, new classes of PNAs that are more readily synthesized, have improved solubility in water, and have better cellular uptake would be welcome among researchers. Any new type of PNA should ideally retain the excellent oligonucleotide-binding properties of the original *aeg*PNA. Unfortunately, numerous chemical modifications to the *aeg*PNA backbone have demonstrated that small changes typically have detrimental effects on binding to complementary DNA and RNA sequences.[2] For example, the tertiary amides in the backbone are crucial for binding. Nielsen's pioneering work in this area demonstrated that changing one tertiary amide in the *aeg*PNA backbone to a tertiary amine dramatically reduces the ability of the oligomer to bind DNA (Figure 1, B).[3] This work indicated that even the presence of favorable ionic interactions between the protonated amine and the oligonucleotide were not able to overcome the loss in structural rigidity. The secondary amides in an *aeg*PNA backbone have been modified to a tertiary *N*-methyl amide, although moderate loss in binding to DNA is observed (Figure 1, C).[4] The work of Efimov elegantly showed that the secondary amides of *aeg*PNA can be replaced with a phosphonate or phosphonamide, again with moderate reduction in binding to DNA (Figure 1, D).[5]

On the basis of the work of Lynn and co-workers with modified nucleosides that have an amine at the 5′ end and an aldehyde at the 3′ end,[6] a PNA analogue with the same

(1) (a) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497. (b) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Nordén, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566. (c) Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624.

(2) Ganesh, K. N.; Nielsen, P. E. *Curr. Org. Chem.* **2000**, *4*, 931.

(3) Hyrup, B.; Egholm, M.; Buchardt, O.; Nielsen, P. E. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1083.

(4) Haaima, G.; Rasmussen, H.; Schmidt, G.; Jensen, D. K.; Kastrup, J. S.; Stafshede, P. W.; Nordén, B.; Buchardt, O.; Nielsen, P. E. *New J. Chem.* **1999**, *23*, 833.

(5) (a) Efimov, V. A.; Choob, M. V.; Buryakova, A. A.; Kalinkina, A. L.; Chakhmakhcheva, O. G. *Nucleic Acids Res.* **1998**, *26*, 566. (b) Efimov, V.; Choob, M.; Buryakova, A.; Phelan, D.; Chakhmakhcheva, O. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20*, 419.

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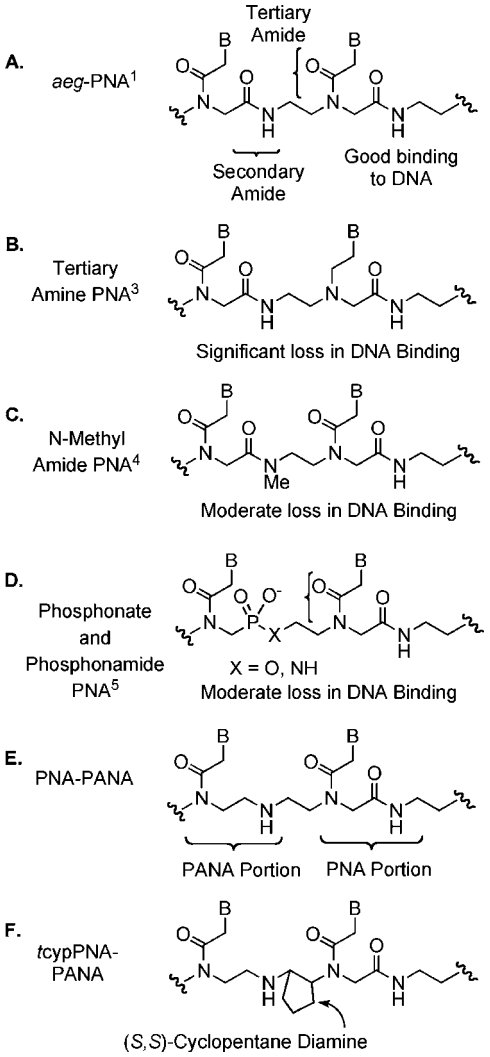


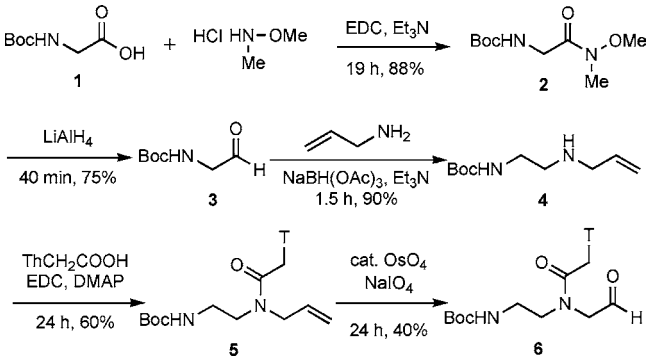
Figure 1. *aeg*PNA and associated backbone modifications.

functional groups could be rapidly assembled by reductive amination in the presence of a DNA template. The ability to access PNAs via DNA templates could significantly enhance access to vast libraries of PNAs that could be screened for diverse functions such as optimal antisense activity, binding to biological targets, or catalysis. We are interested in examining the properties of modified PNAs where secondary amines in the backbone replace secondary amides. We refer to this type of oligomer as a polyamine nucleic acid (PANA). In the course of our research, Liu and co-workers showed that DNA-templated PNA synthesis is possible, provided that tetramer PNA units are coupled on the DNA template.[7] In the Liu PNAs, the effects of the secondary amines on oligonucleotide binding have not been investigated. On the basis of Nielsen's DNA binding studies with tertiary amine PNA (Figure 1, B),[3] increases in the flexibility of the PNA backbone are likely to decrease oligonucleotide binding. Therefore, we predicted that a PNA with one PANA unit (Figure 1, E) would bind very poorly to DNA compared to the original, unmodified PNA.

Most of our research has focused on incorporating *trans*-cyclopentane units into the *aeg*PNA backbone. Replacing the ethylenediamine portion of a PNA backbone with (*S*,*S*)-*trans*-1,2-cyclopentane diamine leads to increases in the melting temperature (T_m) of PNA−oligonucleotide duplexes and triplexes.[8] Therefore, the benefits of a cyclopentane modification could potentially restore losses in binding affinity that may occur in PNAs modified with secondary amines. To test this idea, PNAs in which one of the secondary amides was reduced to a secondary amine were made (Figure 1, E). Once we examined the loss in oligonucleotide binding by introducing a secondary amine into a PNA, we then planned to determine whether binding could be restored to the original levels with incorporation of a cyclopentane into a PNA−PANA oligomer (Figure 1, F). A similar strategy (using hydroxyproline) has been successfully used by Efimov and co-workers to increase the stability of phosphonate PNAs.[9]

To test our hypothesis, monomer **6** was prepared so that a secondary amine in the backbone could be formed via reductive amination (Scheme 1). The synthesis of **6** began

Scheme 1. Synthetic Sequence to Prepare Aldehyde−PNA Monomer



by making Weinreb amide **2** from commercially available Boc-protected glycine (**1**) and *N*,*O*-dimethylhydroxylamine hydrochloride. Conversion of **2** to Boc-glycinal (**3**) proceeded cleanly via reduction with $LiAlH_4$. A reductive amination utilizing sodium triacetoxyborohydride afforded **4**, which was subsequently coupled to thymine carboxylic acid to produce **5**. Dihydroxylation of **5** with osmium tetroxide followed by oxidative cleavage of the intermediate diol using sodium periodate provided **6** in satisfactory yields as a stable solid.[7]

A well-known base sequence was used to examine the effects of the PANA linkage: H_2N-GTAGA***TC***ACT-Lys.

(6) (a) Luo, P.; Leitzel, J. C.; Zhan, Z.-Y. J.; Lynn, D. G. *J. Am. Chem. Soc.* **1998**, *120*, 3019. (b) Li, X.; Zhan, Z.-Y. J.; Knipe, R.; Lynn, D. G. *J. Am. Chem. Soc.* **2002**, *124*, 746.

(7) Rosenbaum, D. M.; Liu, D. R. *J. Am. Chem. Soc.* **2003**, *125*, 13924.

(8) Myers, M. C.; Witschi, M. A.; Larionova, N. V.; Franck, J. M.; Haynes, R. D.; Hara, T.; Grajkowski, A.; Appella, D. H. *Org. Lett.* **2003**, *5*, 2695.

(9) Efimov, V.; Choob, M.; Buryakova, A.; Phelan, D.; Chakhmakhcheva, O. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20*, 419. (b) Efimov, V. A.; Klykov, V. N.; Chakhmakhcheva, O. G. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22*, 593.

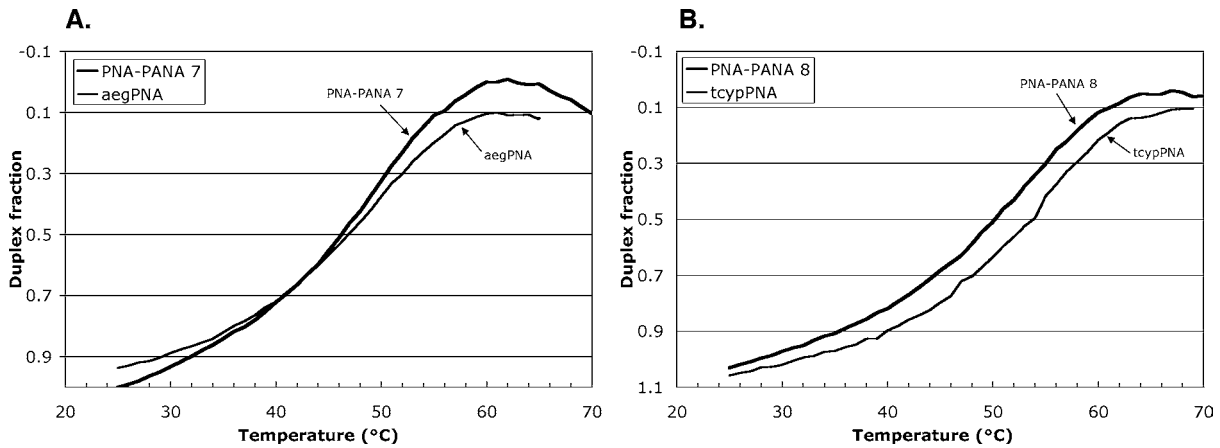
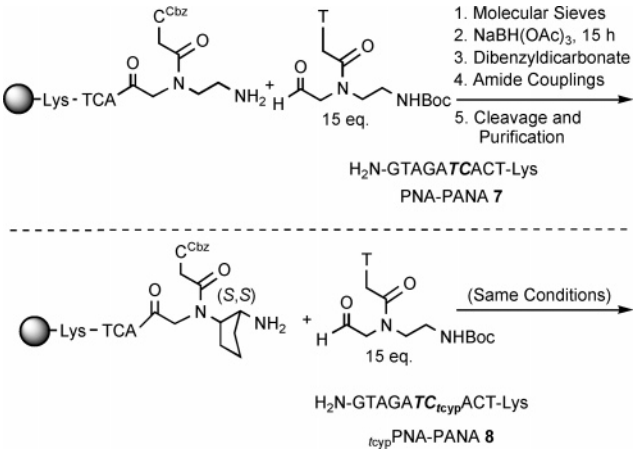


Figure 2. Calculated duplex fraction of PNA−PANA and PNA hybridized to complementary antiparallel DNA. (A) PNA−PANA **7** vs the corresponding *aeg*PNA (note that these two curves are nearly completely overlapped). (B) PNA−PANA **8** vs the corresponding PNA with a cyclopentane in the same position (tcypPNA).

The *aeg*PNA with this sequence has been examined for binding to complementary DNA,[10] and Nielsen used the same sequence to examine the effects of a tertiary amine on DNA binding (in Nielsen's work, the italicized *T* had the tertiary amine).[3] The PANA linkage was placed between the italicized ***TC*** in order to have an analogue that was similar to Nielsen's tertiary amine PNA so that results could be compared.

The synthesis of PANA oligomers requires a reductive amination on solid support between a primary amine of an oligomer on the resin and aldehyde **6** (Scheme 2). After much

Scheme 2. Introduction of PANA Unit into a PNA Oligomer



experimentation, we determined that combining exactly 15 equiv of the aldehyde with the free amine of the resin-bound oligomer in the presence of molecular sieves for 30 min, followed by addition of $NaBH(OAc)_3$, provided the desired PANA linkage. In all cases, preformation of the imine on solid support, prior to addition of the reducing agent was optimal. Furthermore, addition of multiple aldehydes to the primary amine as the result of two successive reductive aminations was never observed, even in the presence of excess aldehyde. The secondary amine was then protected as a benzyl carbamate, and the remainder of the oligomer was synthesized using standard amide-forming reactions.[11] These conditions were used to make PNA−PANA oligomers **7** and **8**, each with a single PANA unit in the middle of a PNA oligomer. The corresponding PNA sequences were also made as controls. The preparation of the cyclopentane monomer for this work has been previously described.[8]

To our surprise, UV melting curves of PNA−PANAs **7** and **8** show little deviation in oligonucleotide binding compared to the control PNAs. Even though we predicted PNA−PANA **7** to have very weak binding to complementary oligonucleotides compared to the *aeg*PNA, the melting curves looked almost identical (Figure 2).[12]

The T_m calculated from the first derivatives of the PNA−PANA:DNA melting curve is identical to the T_m for the *aeg*PNA:DNA duplex (Table 1, entries 1 and 3). In contrast, Nielsen reported a significant reduction in T_m for the tertiary amine PNA (entry 5).[3] Introduction of an (*S*,*S*)-*trans*-cyclopentane diamine into the PNA−PANA resulted in T_m increases that are consistent with the benefits that this ring has on the corresponding *aeg*PNA sequence (entries 2 and 4).

In summary, the PANA linkages in **7** and **8** are tolerated within the complexes of both DNA and RNA. While the rigidity of the tertiary amide is crucial for *aeg*PNA to bind an oligonucleotide,[3] our work indicates that the secondary amide is far less important, at least in the context of the

(10) Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P. E.; Nordén, B.; Graslund, A. *J. Am. Chem. Soc.* **1996**, *118*, 5544.

(11) (a) Koch, T.; Hansen, H. F.; Andersen, P.; Larsen, T.; Batz, H. G.; Otteson, K.; Orum, H. *J. Pept. Res.* **1997**, *49*, 80. (b) Braasch, D. A.; Corey, D. R. *Methods* **2001**, *23*, 97. (c) Nielsen, P. E. In *Peptide Nucleic Acids*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 2002; Vol. 208, pp 3−59.

(12) Fraction of PNA−DNA duplex reported at each temperature in Figure 2 was calculated as described by Breslauer and co-workers: Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 1601.

Table 1. T_m of PNA−PANA:Oligonucleotide and PNA:Oligonucleotide Duplexes[a]

entry	sequences	T_m(DNA)[b]	ΔT_m[c]	T_m(RNA)[b]	ΔT_m[c]
1. PNA−PANA **7**	H_2N-GTAGA**TC**ACT-Lys	48.9	0	55.4	+0.4
2. PNA−PANA **8**	H_2N-GTAGA**TC**$_{tcyp}$ACT-Lys	52.6	+3.1	58.7	+3.9
PNA controls					
3. *aeg*PNA[d]	H_2N-GTAGATCACT-Lys	48.9		54.8	
4. tcypPNA	H_2N-GTAGAT**C**$_{tcyp}$ACT-Lys	54.2	+4.5	59.8	+5.0
tertiary amine PNA					
5. ethPNA[e]	H_2N-GTAGA**T**$_{ethT}$CACT-Lys	26.0	−23.5	38.0	−16.8

[a] DNA and RNA complementary sequences are antiparallel binding strands; d(CATCTAGTGA), r(CAUCUAGUGA). [b] T_m data are reported in units of °C. Approximate error on T_m values is ±0.6 °C. [c] ΔT_m data are referenced against aegPNA (entry 3). [d] T_m agrees with the published value (see ref 10).[e] Data published by Nielsen and co-workers (see ref 3).

sequence studied. The incorporation of a *trans*-cyclopentane constraint in **8** indicates that backbone rigidification can enhance oligonucleotide binding in a PNA−PANA oligomer, as we have demonstrated previously for *aeg*PNA.[8] Access to PNA−PANA oligomers with multiple amine linkages has already been demonstrated in the work of Liu and co-workers using DNA-templated synthesis.[7] We speculate that the success of this approach could be due in part to the compatibility of the PANA linkage within the PNA backbone. Our future work will focus on exploring the extent to which PANA linkages can replace the secondary amides of an *aeg*PNA and comparing our procedures for solid-phase synthesis to DNA-templated synthesis[7] as routes to libraries of these new oligomers.

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Supporting Information Available: Synthetic experimental procedures and characterization data for compounds **2**−**8** and detailed procedures for obtaining T_m for all PNA−PANAs and PNAs. This material is available free of charge via the Internet at http://pubs.acs.org.

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