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A FLEXIBLE AND POSITIVELY CHARGED PNA ANALOGUE WITH AN ETHYLENE-LINKER TO THE NUCLEOBASE: SYNTHESIS AND HYBRIDIZATION PROPERTIES

Birgitte Hyrup, Michael Egholm[§], Ole Buchardt,[†] and Peter E. Nielsen[a,*]

Center for Biomolecular Recognition, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

[a]Department of Biochemistry B, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark. Fax +45 31 39 60 42; Email penpanum@biobase.dk

Abstract. The positively charged and relatively flexible PNA unit ethT (Fig. 1) has an ethylene linker between backbone and nucleobase rather than the methylene carbonyl linker normally present in PNA. The synthesis of the corresponding modified monomer, its incorporation into PNA oligomers, and their hybridization properties to DNA are described.

The potential use of oligonucleotide analogues as antisense and antigene agents has prompted the synthesis of a large repertoire of DNA and RNA like molecules.[1-3] Such compounds are usually tested for their biostability and their ability to hybridize to complementary DNA and RNA in a sequence specific manner. The majority of analogues show decreased affinity towards DNA and RNA targets as compared to the corresponding unmodified oligonucleotides. However, a few modified oligonucleotides have been reported to form complexes with DNA and RNA of comparable or slightly higher stability while preserving the sequence specificity.[3] These include 3'-thioformacetals,[4,5] 3'-phosphoramidates,[6,7] hydroxylamines,[8] and amides.[9-11] Except for the 3'-phosphoramidates, these analogues are neutral. This eliminates the electrostatic repulsion present in complexes between natural oligonucleotides. A further increase in stability has been observed for hybrids between positively charged or zwitterionic DNA analogues and unmodified DNA.[12-15]

Peptide Nucleic Acids (PNA) are neutral DNA mimics radically different from natural oligonucleotides.[16-18] The deoxyribose phosphate backbone is replaced by repeating N-(2-aminoethyl)glycine units with the nucleobases (B) attached through methylene carbonyl linkers (**Fig. 1**). PNA forms highly stable complexes with DNA and RNA and the sequence discrimination is as good as or better than in DNA hybrids.[17] The improved hybridization between PNA and DNA has mainly been attributed to the charge-neutrality of PNA.[17] Furthermore, the restricted flexibility imposed by the amide groups is believed to be important for the favorable DNA-mimicking properties of PNA.

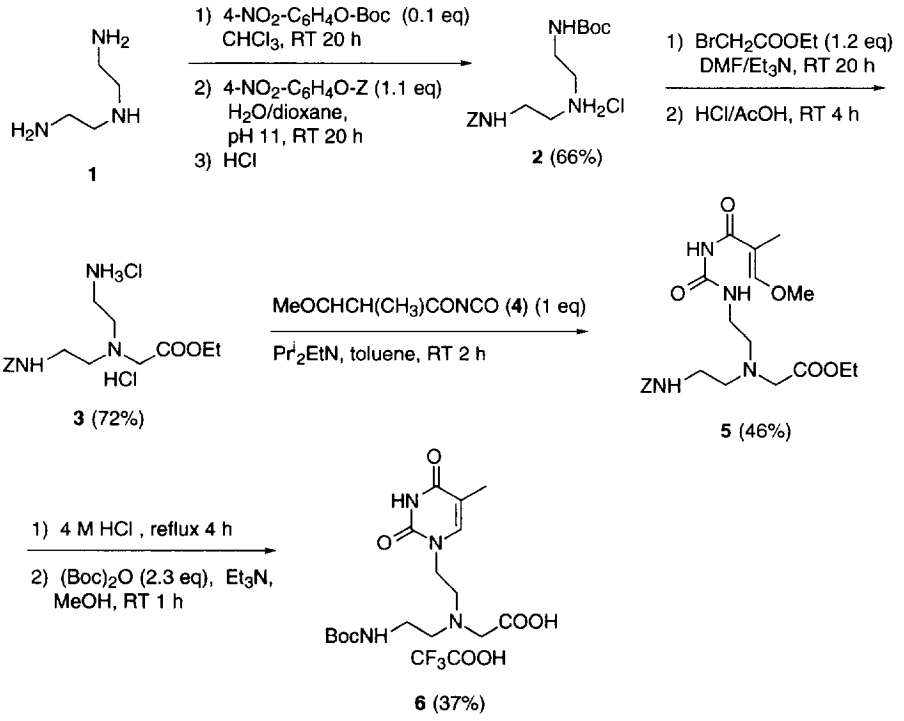
We now report a modification of PNA (ethT, **Fig. 1**) in which the amide linkage to the nucleobase is replaced by a tertiary amine. This modification eliminates the conformational constraint of the amide and introduces a positive charge at the branch points of the PNA backbone at physiological pH. EthT units were incorporated into duplex and triplex forming PNAs and their hybridization properties were evaluated.

Fig. 1

Synthesis

The synthesis of the modified PNA monomer ethT is shown in the **Scheme**. The initial steps involved protection of diethylenetriamine at the primary amines with t-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Z) groups.[19] Subsequent alkylation with ethyl bromoacetate followed by acid treatment afforded **3**. The thymine ring was introduced in a *Shaw* synthesis by reacting the primary amino group of **3** with the acylisocyanate **4**.[20-22] Ring closure, removal of the Z-group, and ester hydrolysis were effected in a single step in boiling hydrochloric acid.[23] The crude product was Boc-protected and purified by reverse phase MPLC.[24]

PNA oligomers incorporating the ethT unit were prepared by solid phase synthesis as previously described.[25] They were purified by reverse phase HPLC and the identity of the oligomers was confirmed by mass spectrometry.[26]



Scheme. *Synthesis of the ethT monomer.*

Hybridization Properties

Duplex motif. A single ethT unit was incorporated into a PNA decamer, H-GTAGA-X-CACT-LysNH$_2$ (X = ethT, **PNA 1**), to study how the modified unit influences the hybridization properties of PNA. **PNA 1** itself gives rise to a small hyperchromicity (apparent T_m of 41°C) which is not fully understood at present. This complicates the interpretation of PNA/DNA hybrid melting curves (especially for mismatched hybrids) and to circumvent the problem we used circular dichroism (CD) to measure the melting, as the CD-signals of PNA oligomers themselves are negligible.[27]

PNA 1 was hybridized to either the complementary antiparallel oligodeoxynucleotide or oligodeoxynucleotides with a single mismatch opposite the modified PNA unit. Representative melting curves are shown in **Fig. 2** and for comparison the melting of the corresponding unmodified PNA/DNA complexes are included (X = T). The melting curves demonstrate that the ethT unit causes a dramatic decrease in duplex stability (ΔT_m = 24°C) while the sequence specificity is preserved (T_m values below 20°C were obtained for T-C and T-T mismatches as well (data not shown)). **PNA 1** also binds to complementary RNA with a T_m of 38°C which should be compared with a T_m value of 56°C for the corresponding unmodified PNA/RNA complex.[28]

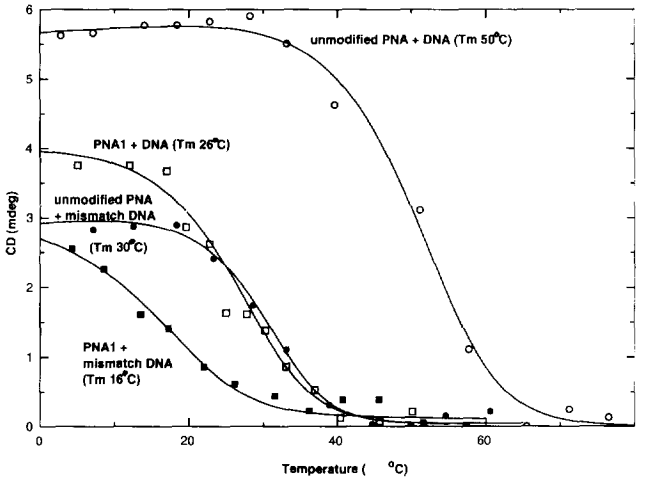
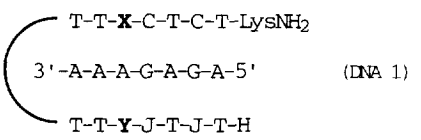


Fig. 2. Melting curves measured using CD.[27] Mismatch DNA has a G opposite the X (T or ethT) in PNA. The solutions were 10 mM in phosphate, 0.1 mM in EDTA, and pH was 7.0.

Triplex motif. A homopyrimidine bis-PNA[29] (**Fig. 3**) was used for the incorporation of ethT into a triplex forming PNA. The modified unit was placed either in the *Watson-Crick* (**PNA 3**) or in the *Hoogsteen* strand (**PNA 4**) or it was present in both strands (**PNA 5**). The modified PNA oligomers were hybridized to complementary DNA under different salt and pH conditions and the thermal stability of the complexes was determined using UV-measurements.[30] The results are summarized in the **Table**. Comparing complexes involving the modified PNA (**3**, **4**, or **5**) with the unmodified complex (**2**) reveals that the ethT unit causes a large destabilization (ΔT_m = 18°C when ethT is in the *Watson-Crick* strand, 20°C when it is in the *Hoogsteen*

strand, and 33°C when it is incorporated into both strands; pH 7, 100 mM NaCl). It seems to be of minor importance whether the ethT unit is present in the *Watson-Crick* or in the *Hoogsteen* strand and the effect of each modification is close to additive in complex **5**.



PNA 2: X = T, Y =T
PNA 3: X = ethT, Y = T
PNA 4: X = T, Y = ethT
PNA 5: X = ethT, Y = ethT

Fig. 3. *Bis-PNAs (the linker between the two strands consists of 3 -$NHCH_2CH_2OCH_2CH_2OCH_2CO$- units); J = pseudoisocytosine.*

Complexes incorporating ethT seem to be no more sensitive to pH than the unmodified complex **2**. Since the PNAs contain the base pseudoisocytosine[29] (J) instead of cytosine in the Hoogsteen strand, the triplex formation is expected to be essentially independent of pH.[29,31] The observed preference for acidic conditions in both modified and unmodified complexes might therefore be caused by electrostatic attraction between the C-terminal lysine and the N-terminal amino groups of PNA and negatively charged DNA. The stability of all complexes is favoured by a low salt concentration as is usually the case for unmodified PNA-DNA complexes.[17] The complexes incorporating ethT do not show stronger sensitivity to the salt concentration than unmodified complexes.

Table. T_m (°C) for bis-PNA/DNA triplexes[a]

		Complexes of DNA 1 with **PNA**			
pH	NaCl (mM)	**2**	**3**	**4**	**5**
5	100	64	47	44	29
7	100	62	44	42	29
9	100	56	41	38	26
7	0	66	49	46	31
7	1000	56	39	36	24

[a] The melting temperatures of the hybrids were determined as previously described.[33] The solutions were 10 mM in phosphate, 0.1 mM in EDTA, and the NaCl concentration and pH were as indicated in the table.

Discussion

The modified PNA unit ethT changes both the conformational flexibility of PNA and its charge. The amide connecting the backbone and the linker to the nucleobase in unmodified PNA is a rigid unit due to the partial double bond character of the C-N bond whereas its replacement in ethT, a tertiary amine, is significantly more

flexible. Although a more flexible unit might still allow for a suitable geometry, the hybridization energy will be less favorable due to a greater loss in entropy as previously observed for acyclic DNA analogues.[33-35] The tertiary amine is expected to be protonated at neutral pH and it might therefore contribute to an electrostatic attraction between PNA and DNA. The magnitude of the attraction should be dependent on the distance between the amine and the phosphates of the DNA backbone. Furthermore, it should be sensitive to changes in pH as well as changes in the ionic strength.

As described above, the ethT unit gives rise to dramatic decreases in the stability of both PNA-DNA duplexes and triplexes. Despite the lower stability, the thymine in ethT seems to specifically recognize the complementary adenine in the DNA strand since mismatches cause a further decrease in T_m.

If the ethT unit contributes significantly to the electrostatic attraction between PNA and DNA this should be reflected in the relationship between T_m and pH. Although the complexes are more stable at pH 5 than at pH 9, the difference in T_m is negligible relative to the unmodified complex **2**. The same pH dependency is observed when no sodium chloride is added (data not shown) suggesting at the most a very weak electrostatic attraction between ethT and DNA. The salt dependence of the T_m is also similar to that observed for the unmodified complex indicating that any additional electrostatic attraction between PNA and DNA is not shielded by salt.

The decrease in T_m for complex **5** is 33°C relative to the unmodified complex **2** (pH 7, 100 mM NaCl). Since this decrease in T_m corresponds almost to the sum of the decreases observed for complex **3** and **4** (18 and 20°C, respectively), this might indicate that any unfavorable geometry imposed by the ethT unit in one PNA strand on the DNA does not affect the binding of the second PNA strand of the triplex.

Conclusion

The PNA unit ethT has been shown to cause a large destabilization of both PNA/DNA duplexes and $(PNA)_2$/DNA triplexes. Nevertheless, there seems to be Watson-Crick base pairing between the thymine of ethT and adenine in complementary DNA since mismatches opposite to ethT cause a further decrease in T_m. The lower stability of PNA-DNA complexes incorporating the ethT unit is most probably due to its higher flexibility. These results emphasize the importance of the amide function(s) in the DNA-mimicking PNA structure, contributing rigidity to the backbone.

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References and Notes

§ Present address: PerSeptive Biosystems, Inc., Framingham, Massachusetts, USA.
† Deceased.
* To whom correspondence should be addressed.

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