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NEW HETERO-OLIGOMERIC PEPTIDE NUCLEIC ACIDS WITH IMPROVED BINDING PROPERTIES TO COMPLEMENTARY DNA

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Dedicated to Prof. Peter Welzel on the occasion of his 60th birthday

Abstract: Hetero-oligomeric PNAs consisting of new monomeric building blocks and various amounts of N-(2-aminoethyl) glycine have been synthesized by solid-phase chemistry. Some of these new compounds show stronger binding to complementary DNA than the original PNAs, and are consequently very interesting candidates as antisense compounds for applications in therapy and in diagnostics.

Antisense oligonucleotides are of great interest as potential molecular biological tools and therapeutic agents.[1] They have the ability to interact with a complementary sequence on mRNA[2] and to inhibit specific gene-expression and protein biosynthesis. Natural antisense oligonucleotides have been employed successfully for the inhibition of viral replication, but their low stability in biological media[3] has limited their use as new therapeutic agents. This problem can be solved by the use of modified non-natural analogues and derivatives with improved nuclease resistance. Some of these compounds have been approved for clinical trails within the last years.[4] Since their discovery some years ago, PNAs[5] have become very attractive compounds for antisense research. This is due to their remarkable affinity to complementary sequences of DNA and RNA by Watson-Crick base pairing. Since they have no phophorodiester bridge in their backbone, they are not substrates for degradation by nucleases. They hybridize selectively to their target mRNAs without non-specific interactions which can lead to unfavourable side-effects, as reported for the phosphorothioates.[6]

In this paper, we wish to describe the synthesis and the properties of hetero-oligomeric sequences of new PNA-building blocks in combination with the known N-(2-aminoethyl) glycine (aeg).[7] We have synthesized a range of monomeric building blocks for PNA-synthesis. Unfortunately, after oligomerisation all these compounds have shown no detectable hybridization to the complementary DNA-sequences. Based on published data[8] indicating that aeg exhibits a tendency to pre-organisation in helical structures, we synthesized heterooligomeric compounds containing our new monomers and various amounts of aeg. We then studied the hybridization properties, the resistance to enzymatic degradation, and the double strand displacement of these modified PNAs.

The monomeric building blocks were prepared as previously described.[9] The oligomeric compounds were synthesized using a peptide synthesizer[10] and standard Boc-chemistry on PAM- or MBHA-resins. In order to suppress the tendency for aggregation in thymine PNA oligomers and to avoid base-migration, firstly Boc-Lys(2-chloro-Z)-OH was coupled to the resin by activation with hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) in NMP. The Boc-group was removed by TFA and the resin washed to

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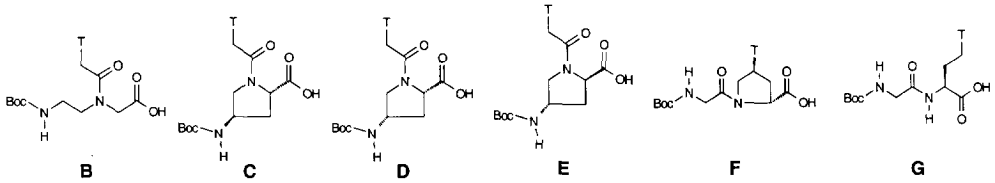
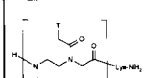
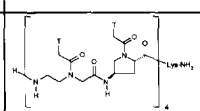
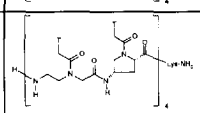
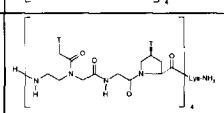
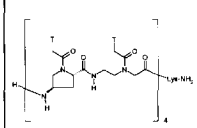
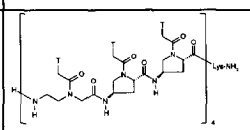
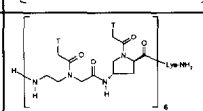
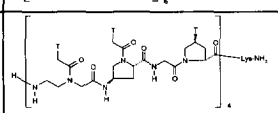
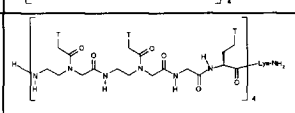
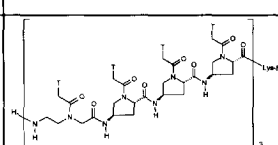
neutral pH with diisopropylethylamine. The building blocks were then incorporated into oligomers using the same coupling procedure. The sub-units **B - G** (shown in figure 1) of the oligomers are different with respect to their backbones; **B** contains the known N-(2-aminoethyl) glycine (aeg), **C** contains L-4-trans-amino-proline, **D** contains L-4-cis-amino-proline, **E** is based on D-4-trans-amino-proline, **F** and **G** are a combination of glycine with α–amino acids containing nucleobases, easily prepared by standard peptide chemistry.

Figure 1. Sub-units of the synthesized PNAs



At the end of the synthesis the compounds were cleaved from the resin by HF and anisole at 0°C. Remaining anisole was removed by washing with ether. The oligomer was extracted with 30% acetic acid and the polymer support was filtered off. The resulting filtrate was lyophilized and purified by reverse-phase HPLC using a gradient of TFA in water and TFA in water/acetonitril.[11] The oligomers were characterised by mass-spectroscopy using LDI-methods.

Table 1. T_m (°C) of the modified oligonucleotides with complementary DNA

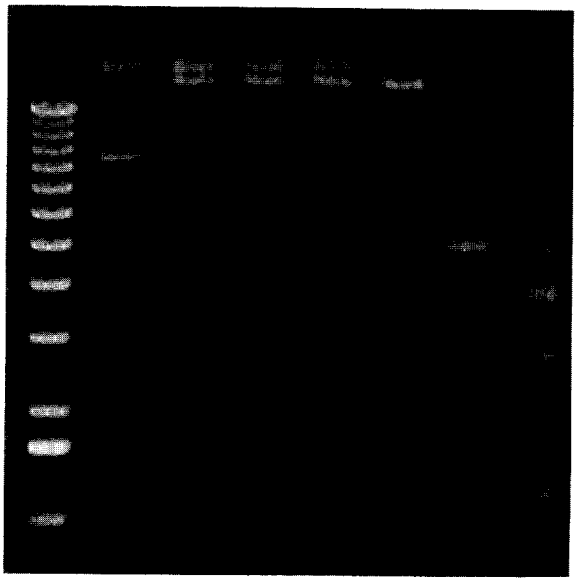
No.	sequence	length	yield (%)[b]	T_m-value (°C)
1		8	[c]	40.0-42.0
2		8	61.2	47.0
3		8	34.9	27.0
4		8	67.0	33.0
5		8	76.6	46.0
6		12	13.7	48.8
7		12	91.0	27.0
8		12	21.0	50.0-52.0
9		12	3.0	28.5
10		12	18.4	34.4

[a] DNA synthesized using standard phosphoramidite technology on an Applied Biosystem ABI 380B® synthesizer; [b] after purification by HPLC (purity >95%); [c] not determined

To study the hybridizing properties of the modified oligomers with their complementary DNA, melting temperatures (T_m-values) were determined by UV-measurements.[12] The compounds show well-defined single-phased melting profiles. The T_m values and the sequences are listed in Table 1. In our hands the combination

of aeg (**B**) with L-4-trans-amino-proline (**C**) shows stronger binding properties to complementary DNA than homo-oligomers of aeg. Best results were obtained with a 1:1 mixture of building blocks **B** and **C** as shown by T_m-values for the hybridisation of compounds **2** or **5** with their complementary DNA. Compared with the original PNAs, they showed a 6-7°C increase in T_m. These combinations appear promising materials for the generation of new antisense oligonucleotides. Oligomers containing the D-trans isomer **E** resulted in a large decrease in hybridisation compared with oligomers containing the analogue **C**. The incorporation of the other sub-units (**D, F, G**) also dramatically reduced the binding to A_{12} or A_8 oligomers. The duplexes formed between these compounds and DNA were significantly destabilized.

Figure 2. Agarose gel electrophoretic analysis of compound **2** mediated strand displacement in double stranded plasmid DNA[13]



The L-4-trans-amino-proline containing PNAs hybridize in a sequence-selective manner to double stranded DNA. Hybridization and strand displacement in double stranded plasmid DNA is shown for these oligomers (compound **2, 5, 6, 10**) by incubation in samples containing plasmid DNA and single-strand DNA recognizing S1-nuclease. For example, compound **2** binds sequence-selectively to complementary target sequences within coiled plasmid DNA. Specific single strand displacement of double stranded plasmid DNA results in generation of characteristic DNA fragments by S1 nuclease digestion. Analysis of formed DNA fragments is conducted by agarose gel electrophoresis as shown in Fig. 2. The size of all detectable DNA fragments (i.e. 4880, 3820, 2670, 2210, 1150, and 1060 base pairs) is consistent with the expected linearization of the plasmid (4880 base pairs) and partial cleavage of plasmid DNA at the target sites for binding of compound **2**.

The same pattern of S1-nuclease generated fragmentation of plasmid DNA can be obtained using compound **6** or **10**. Stoichiometric amounts of plasmid DNA and octameric PNA carrying alternating aminoproline and N-(2-aminoethyl) glycyl building blocks, resulted in detectable and specific S1 nuclease mediated DNA fragmentation under the assay conditions used. Addition of non-complementary amino-proline containing PNA as a negative control did not result in detectable DNA fragmentation.[14]

We also checked hybridisation of compound **2** in a tris-buffer in a different ratio to the complementary DNA-sequence. Best results were obtained using a ratio of 1 to 0.5, suggesting a 2:1 hybridisation.[15] In further experiments we successively increased the amounts of monomer **C** in ratio to **B** from 1:1 up to 1:2 and 1:3. Again, a loss in hybridisation properties was observed.[16] This suggests that aeg has a strong effect, leading to a structure pre-formed for recognition of DNA. Molecular modelling shows the possibility of forming a helical structure in aeg-polymers stabilized by intramolecular hydrogen bonds between the carbonyl function in the side-chain with the nucleobase and an amide proton of the backbone.[17] Incorporation of L-4-trans-aminoproline can increase binding by forming structures better fitting to DNA-helices.

In conclusion, we have prepared modified PNAs for antisense research. These modified compounds contain various amounts of N-(2-aminoethyl) glycine. They form duplexes with the complementary strands of DNA and have improved base-pairing properties than the previously known PNAs. The new compounds also show excellent stability against enzymatic degradation.

References and Notes

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#current address: Biotest AG, Landsteinerstr. 5, D-63303 Dreieich, Germany.

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7 Abbreviations used: aeg, N-(2-aminoethyl) glycine; Boc, tert.-butoxycarbonyl; Z, benzyloxycarbonyl; TFA, trifluoroacetic acid; NMP, N-methyl-pyrrolidone.

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11 Crude oligomers were purified on a C_{18} columm using a gradient of water (0.1% TFA, buffer A) and water/acetonitrile 7:3 (v/v) (0.1% TFA, buffer B); 0 min: 95% A, 30 min: 40% A; 40 min: 20% A; 45 min: 20% A; 50 min: 95% A.

12 The melting temperatures of the hybrids were determined on an Perkin Elmer 'Lambda Bio'UV-Vis spectrometer using the manufactures method 'PE-Temp'. PNAs and DNAs are dissolved in 700 µl water until an absorption of 0.3 is reached. Both strands are combined, heated to 95°C for 5 min and then cooled to room temperature overnight. The double strands were examined within a temperature range from 20°C to 80°C using the above mentioned method. From the maximum of the first derivate of the plot of absorption versus temperature, the T_m-values were determined.

13 Agarose gel electrophoretic analysis of compound **2** mediated strand displacement in double stranded plasmid DNA. Specific plasmid DNA fragmentation by S1 nuclease digestion of single strand DNA cleavage is detected in samples 6 - 8. Composition of samples in lanes 1 - 8 is as follows. 1: molecular weight marker; 2: 1 µg plasmid DNA; 3: 1 µg plasmid DNA plus 10 U S1 nuclease; lanes 4 - 8 as for lane 3 with increasing amounts of **2** containing 0.0001 µg, 0.001µg, 0.01µg, 0.1 µg, and 1.0 µg respectively.

14 The plasmid used for tests was composed of pSP64 backbone (Promega Corp. Madison, WI USA) carrying a globin polyadenylation sequence and a coding sequence for delta-subunit of torpedo californica nicotinic receptor. Test samples contained 1.0 µg plasmid, 14 µl water, and varying amounts of **2**. The samples were incubated for 45 min at 37°C. Subsequently, 4µl buffer (250 mM sodium acetate, 1 M NaCl, 2.5% glycerol, 5 mM $ZnCl_2$, pH 4.4) and 10 U S1-nuclease (*Aspergillus niger*, Boehringer Mannheim, Germany) were added to the samples and incubated for 15 min at 30°C. The reaction was stopped by cooling on ice and by adding 1 µl 0.5 M EDTA and 3 µl of sample buffer (50% glycerol, 0.25% bromophenol blue in 40 mM tris-HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.2). The reaction mixture was loaded onto an agarose gel (1.2 %) for electrophoresis and subsequent staining by ethidium bromide. Molecular weight markers for DNA fragments were used from Gibco-BRL, Gaithersburg, Maryland, USA.

15 Different ratios were choosen: 0.2:1; 0.25:1; 0.33:1; 0.5:1; 1:1; 2:1 and 3:1 DNA to PNA. Probes (45 λM) in tris*HCl (5 mM, 0.1 ml) were heated up to 93°C for 5 min and slowly cold to room temperature. The resulting hybrids were characterised by dynamic gel capillar electrophoresis (Rose, D. J. *Anal. Chem.*, **1993**, *65*, 3545). The yield of the hybridisationproducts as a function of the concentration of complementary $d(A)_8$ shows a rapid increase up to a ratio of 0.5:1. Although increasing the ratio of $d(A)_8$ to compound **2**, the hybridisationproduct remains relatively constant, but more and more free DNA is detected.

16 The T_m-value for a 12 mer is reduced from 48.8°C (compound **6**) to 34.4°C (compound **10**) by incorporation of the sub-unit **C** in a ratio of 1:3 instead of 1:2. Both T_m-values are low compared with a T_m-value of 47.0°C for the 8mer **2** with a 1:1 ratio of sub-units **B** and **C** (a 12 mer with a 1:1 ratio is not prepared because of the expected high T_m-value (>80°C) of this compound).

17 This point is some what controversial, see: Chen, S.-M.; Mohan, V.; Kiely, J. S.; Griffith, M. C.; Griffey, R. H. *Tetrahedron Lett.* **1994**, *35*, 5105 and Torres, R. A.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A* **1996**, *93*, 649.

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