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Optimization of the Binding Properties of PNA-(5')-DNA Chimerae

A.C. van der Laan, P. Havenaar, R.S. Oosting[‡], E. Kuyl-Yeheskiely, E. Uhlmann[#] and J.H van Boom[*]

Leiden Institute of Chemistry, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA, Leiden. The Netherlands
[‡]Solvay Duphar B.V., Department of Biotechnology, P.O. Box 900, 1380 DA, Weesp. The Netherlands
[#]Hoechst Marion Roussel Deutschland GmbH, Chemical Research, G 838, D-65926 Frankfurt. Germany

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Abstract: The synthesis and evaluation of PNA-(5')-DNA chimerae containing either a 5'-amide (*i.e.* **1a**), a 5'-phosphodiester (*i.e.* **1b**) or 5'-phosphonate linkages (*i.e.* **1c,d**) at the junction site are described. The 5'-linkages could be installed using either 5'-amino-5'-deoxythymidine phosphoramidite **2**, *O*-[2-(2-aminoethyl)-(thymin-1-ylacetyl)amino]ethyl phosphoramidite **3**, *N*-(2-aminoethyl)-*N*-(thymin-1-ylacetyl)aminomethyl phosphonate **4** or *N*-(2-aminoethyl)-*N*-(allyloxycarbonyl)aminomethyl phosphonate **5** as building blocks, respectively. It is shown that PNA-(5')-DNA of type **1a-c** have a higher binding affinity with complementary RNA than native DNA, and that the antisense activity is mainly due to RNase H.

Introduction

The strong and sequence specific hybridization of PNA[1] to complementary DNA and RNA was an incentive for the design of potential therapeutics[2] and diagnostics.[3] Despite the promising outlook, the efficacy of PNA as antisense agents is strongly diminished by its low water solubility,[4] and the fact that the RNA in PNA•RNA duplexes is not cleaved by the enzyme RNase H.[5] In order to circumvent these shortcomings, much effort has been focused on conjugates of PNA and DNA (*i.e.* PNA/DNA chimerae).[6]

As part of a program dealing with the design and synthesis of potentially useful antisense probes, we recently reported a fully automated on-line solid-phase synthesis of PNA/DNA chimerae.[6] It was shown that DNA-(3')-PNA, having a phosphodiester linkage between PNA and the 3'-end of DNA, forms more stable duplexes with complementary DNA than natural DNA.[6a] In contrast, PNA-(5')-DNA (*i.e.* **1a**, Figure 1) and PNA-(5')-DNA-(3')-PNA chimerae, containing a rigid 5'-amide linkage between the PNA and DNA, have a lower binding affinity with the DNA target.[6b] NMR studies revealed that helices of PNA duplexes with DNA are wider (*i.e.* 23Å in diameter) and have a larger helical turn (*i.e.* 13 base pairs)[7] than native helices of DNA•DNA duplexes. As a consequence, these differences in helices may cause some distortion in duplexes of PNA/DNA chimerae with DNA, especially if the PNA part is attached to DNA *via* a conformationally restricted linker. We reasoned that the decrease in duplex stability of PNA-(5')-DNA and PNA-(5')-DNA-(3')-PNA with DNA targets may be attributed to a distortion in the duplex. It is not excluded that this distortion is somewhat more enhanced due to the presence of the rigid 5'-amide linkage.

With the objective to optimize the hybridization properties of PNA/DNA conjugates, we here report the synthesis and evaluation of PNA-(5')-DNA (*i.e.* **1a-c**, Figure 1) and PNA-(5')-DNA-(3')-PNA chimerae containing either a rigid 5'-amide bond, a more flexible 5'-phosphodiester or a 5'-phosphonate linkage at the junction site. We also investigated a PNA-(5')-DNA chimera (*i.e.* **1d**) having a linker comprising of a 5'-

*e-mail: Boom_j@chem.leidenuniv.nl; Fax: ++ 31 (0)71-5274307

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phosphonate bond lacking a nucleobase. It was assumed that the latter type of linker would have a beneficial effect on the binding properties of the chimerae.

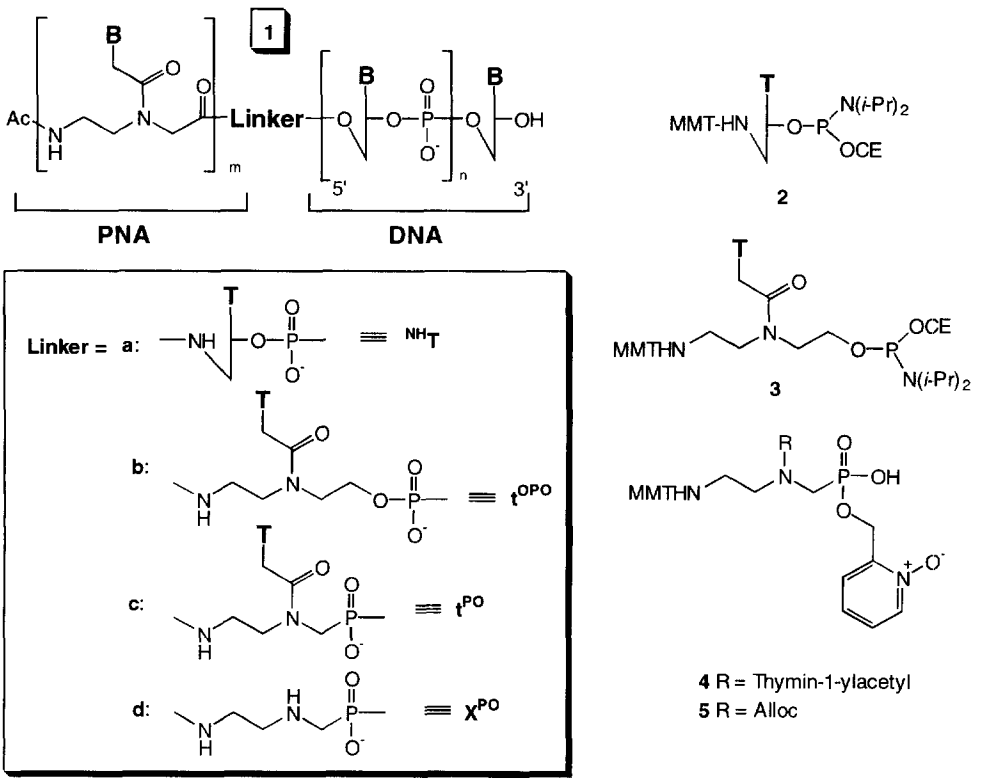


Figure 1: Primary structures of the four different types of PNA-(5')-DNA chimerae.

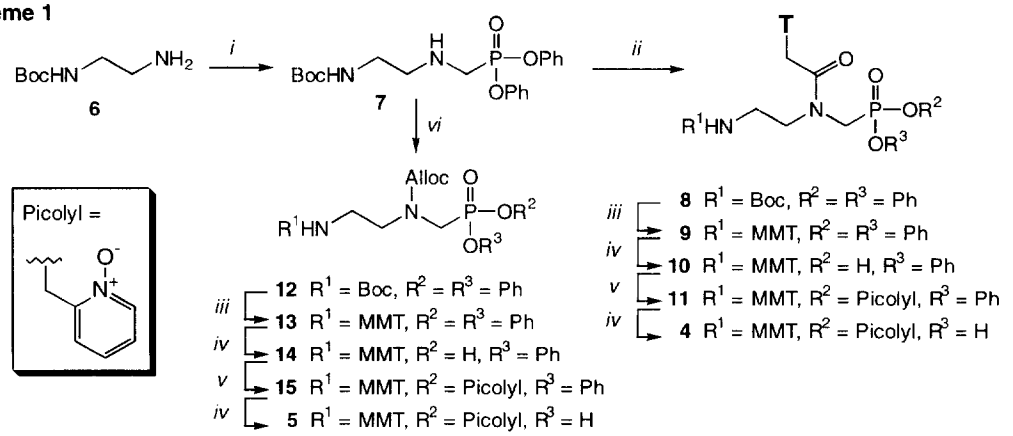
Results and discussion

Retrosynthetic analysis of the target compounds **1a** and **1b** reveals that 5'-amino-5'-deoxythymidine phosphoramidite **2**[8] and *O*-[2-(2-aminoethyl)-(thymin-1-ylacetyl)amino]ethyl phosphoramidite **3**[9] are suitable building blocks for the introduction of the 5'-amide and 5'-phosphodiester linkages, respectively. On the other hand, the 5'-phosphonate linkages in 1c and 1d could be installed using either *N*-(2-aminoethyl)-*N*-(thymin-1-ylacetyl)aminomethyl phosphonate 4 or *N*-(2-aminoethyl)-*N*-(allyloxycarbonyl)aminomethyl phosphonate 5.

The preparation of the requisite phosphonate building blocks 4 and 5 could be realized by the sequence of reactions depicted in Scheme 1. Condensation of *t*-butyloxycarbonyl (Boc) protected ethylenediamine 6 with a slight excess of formaldehyde, followed by phosphonylation of the resulting imine with an equimolar amount of diphenyl phosphite led to the quantitative formation of diphenyl phosphonate derivative 7.[10] Coupling of thymin-1-yl acetic acid to crude backbone 7 under the influence of dicyclohexylcarbodiimide (DCC) furnished, after purification, fully protected thyminyl derivative 8. Removal of the temporary Boc protecting group with trifluoroacetic acid (TFA), and subsequent tritylation of the resulting primary amino function with MMT-Cl afforded phosphonate 9. Transformation of diester 9 into monomer 4, bearing the catalytic 1-oxido-pyridine-2-methyl (Picolyl) phosphonate protecting group,[11] was realized by executing the following sequence of

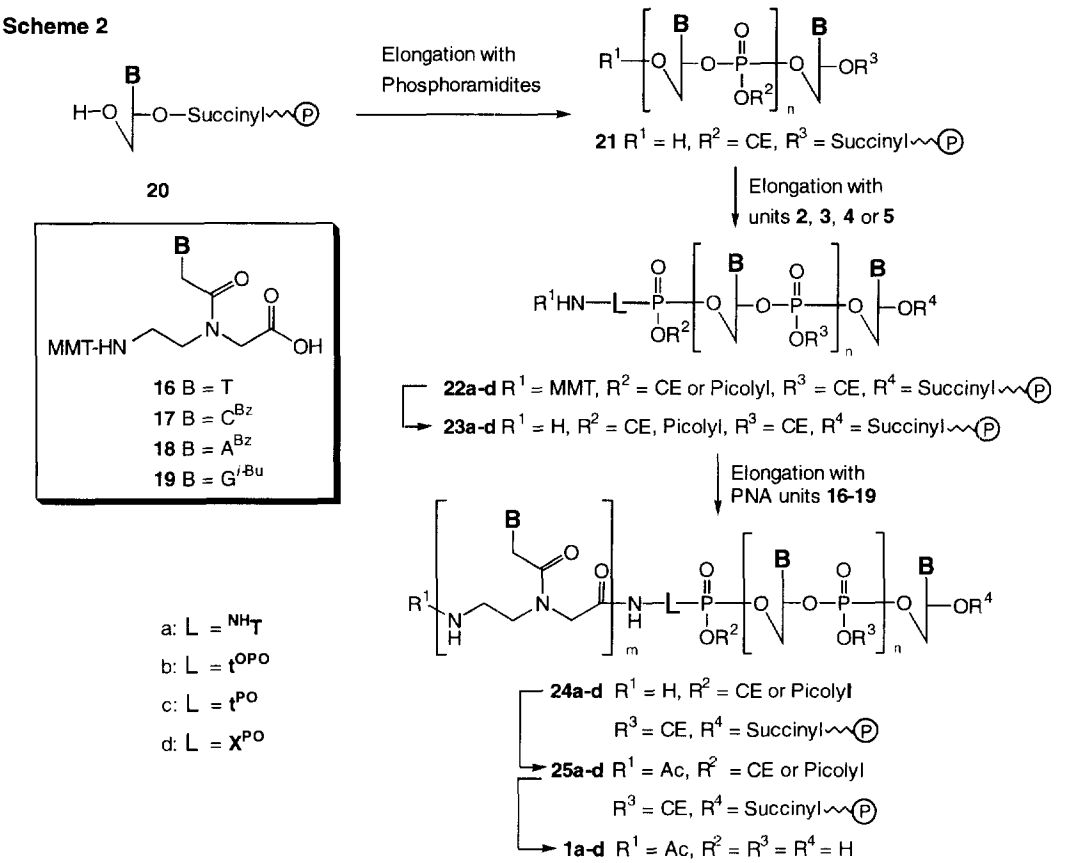
reactions. Removal of one phenyl group in 9 under the influence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in the presence of H_2O yielded the corresponding phosphonate monoester 10. Condensation of 10 with 1-oxido-2-pyridyl methanol under the agency of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) and 4-methoxy-1-oxido-pyridine led to phosphonate diester 11. Finally, selective removal of the phenyl group in 11 with DBU-H_2O proceeded smoothly to yield picolyl phosphonate 4. Similarly, intermediate 12, obtained after treatment of amine 7 with allyl chloroformate, was transformed into building unit 5.

Scheme 1



Reagents and conditions: (*i*) a. CH_2O in EtOAc, 15 min; b. $HP(O)(OPh)_2$ in toluene, 65°C, 30 min; (*ii*) Thymin-1-yl acetic acid, DCC in DMF, 2 h; (*iii*) a. 50% TFA in CH_2Cl_2, 30 min; b. MMT-Cl, pyridine in CH_2Cl_2, 4 h; (*iv*) DBU in CH_3CN/H_2O (95/5, v/v), 15 min; (*v*) Picolyl-OH, 4-methoxypyridine-1-oxide, TPS-Cl in CH_3CN/pyridine (4/1, v/v), 30 min; (*vi*) Allyl chloroformate, DiPEA in CH_2Cl_2, 3 h.

The target PNA-(5')-DNA chimerae **1a-d** were assembled, as outlined in Scheme 2, using a fully automated DNA synthesizer. To this end, thymidine unit **20**, immobilized *via* a 3'-*O*-succinyl linkage to controlled pore glass, was extended with cyanoethyl (CE) DNA phosphoramidites, to give immobilized DNA **21**. In order to synthesize chimera **1a** containing the 5'-amide linkage or chimera **1b** with the 5'-phosphodiester linkage, the free 5'-OH function in **21** was phosphitylated at this stage either with phosphoramidite **2** or **3** following standard DNA synthesis protocols to give, after oxidation of the intermediate phosphite triester, fully protected fragment **22a** (L = ^{NH}**T**, R^2 = CE) or **22b** (L = $\mathbf{t}^{OPO}$, R^2 = CE), respectively. Acidolysis of the MMT group in CPG bound **22a-b** and elongation of intermediates **23a-b** with PNA monomers **16-19**[12] as described previously[6b] gave, after detritylation, the fully protected PNA-DNA **24a-b**. Acetylation of the resulting primary amino function in **24a-b** led to anchored PNA-(5')-DNA **25a-b**. Finally, cleavage of chimerae **25a-b** from the solid support and concomitant removal of the base labile protecting groups was effected with methanolic ammonia, to give crude PNA-DNA conjugate **1a-b**. In a similar fashion, chimerae **1c,d** containing the respective 5'-phosphonate linkages $\mathbf{t}^{PO}$ and $\mathbf{X}^{PO}$ were assembled. In this case, the 5'-phosphonate linkages were installed[11] by phosphonylation of the free 5'-hydroxyl in **21** with phosphonate **4** or **5** under the agency of TPS-Cl yielding fully protected intermediate **22c** (L = $\mathbf{t}^{PO}$, R^2 = Picolyl) or **22d** (L = $\mathbf{X}^{PO}$, R^2 = Picolyl), respectively. It is important to note that prior to the ammonia treatment of **25d** (→ **1d**), the allyloxycarbonyl (Alloc) group in **25d** was removed under the influence of Pd(0).[13] The PNA-(5')-DNA-(3')-PNA chimerae having different 5'-linkers (entry 11-13, Table 1) were synthesized as reported previously.[6b] The mass of all



chimerae in Table 1 were confirmed, after purification by RP-HPLC, by MALDI-TOF MS spectrometry.

The binding affinity of the PNA/DNA chimerae with complementary DNA and RNA was investigated by measuring the melting temperatures (T_m) of the corresponding duplexes. It can be seen (Table 1) that duplexes of PNA-(5')-DNA with the complementary DNA strand (entries 3-5 and 8-11) have a considerable lower stability, presumably due to distortion of the helices, than natural DNA•DNA duplexes (entries 1, 6). This destabilizing effect is even more pronounced in the case of PNA-(5')-DNA-(3')-PNA chimerae (entries 12-14), a drop of 11-14°C in T_m was observed compared with the DNA•DNA duplexes. However, duplexes of PNA-(5')-DNA **1a-c** (entries 3-5 and 8-10) with the RNA target showed a significantly higher stability than the DNA•RNA duplexes, indicating that PNA-(5')-DNA chimerae accommodate more easily in an RNA helix than in a DNA helix.[14] In the case of the PNA-DNA conjugate **1d**, containing the flexible linker $\mathbf{X}^{PO}$, the stability of the duplexes with both DNA and RNA decreased drastically (entry 11). The destabilizing effect of this type of PNA-(5')-DNA strongly indicates that the increase of flexibility between PNA and DNA has a negative effect on Watson-Crick base pairing. It can also be seen (entries 4, 9) that chimerae containing a 5'-phosphodiester linkage have a lower binding affinity with nucleic acids than the corresponding PNA/DNA conjugates having a 5'-amide or 5'-phosphonate linkage. The decrease in duplex stability is probably due to the presence of two additional atoms in the backbone. On the other hand, it is evident that the 5'-phosphonate linkage $\mathbf{t}^{PO}$ (*i.e.* **1c**, entries 5 and 10) is the most suitable linker of connecting PNA to the 5'-end of DNA. This assumption is

endorsed by the fact that the decrease in duplex stability (entry 13) of PNA-(5')-DNA-(3')-PNA chimera containing the 5'-phosphonate linker t^{PO} with both DNA and RNA is less dramatic (*cf.* entries 12, 13).

Entry	PNA/DNA	Sequence[15]	DNA T_m (ΔT_m)	RNA T_m (ΔT_m)
1	DNA	TAC CTA ATT AGA CGG T	53.7 °C	51.4 °C
2	PNA	^{Ac}tac cta att aga cgg t	59.7 °C (+6.0)	67.0 °C (+15.6)
3	PNA-5'-DNA	^{Ac}tac cta at ^{NH}T AGA CGG T	50.5 °C (-3.2)	54.5 °C (+3.1)
4	PNA-5'-DNA	^{Ac}tac cta a $\mathbf{t}^{OPO}$ TAG ACG GT	47.5 °C (-6.2)	52.1 °C (+0.7)
5	PNA-5'-DNA	^{Ac}tac cta a $\mathbf{t}^{PO}$ TAG ACG GT	50.4 °C (-3.3)	55.5 °C (+4.1)
6	DNA	ATT TCA TCT GCA ACT TCT	57.4 °C	56.6 °C
7	PNA	^{Ac}att tca tct gca act tct	64.3 °C (+6.9)	73.1 °C (+16.5)
8	PNA-5'-DNA	^{Ac}att tca tc ^{NH}T GCA ACT TCT	50.7 °C (-6.7)	58.0 °C (+1.4)
9	PNA-5'-DNA	^{Ac}att tca tc $\mathbf{t}^{OPO}$ GCA ACT TCT	44.0 °C (-13.4)	56.7 °C (+0.1)
10	PNA-5'-DNA	^{Ac}att tca tc $\mathbf{t}^{PO}$ GCA ACT TCT	50.8 °C (-6.6)	59.2 °C (+2.6)
11	PNA-5'-DNA	^{Ac}att tca tc $\mathbf{X}^{PO}$ GCA ACT TCT	39.2 °C (-18.2)	39.7 °C (-16.9)
12	PNA-5'-DNA-3'-PNA	^{Ac}att tca ^{NH}T CT GCA AC t tct	43.0 °C (-14.4)	52.0 °C (-4.6)
13	PNA-5'-DNA-3'-PNA	^{Ac}att tca $\mathbf{t}^{OPO}$ CT GCA AC t tct	42.9 °C (-14.5)	53.2 °C (-3.4)
14	PNA-5'-DNA-3'-PNA	^{Ac}att tca $\mathbf{t}^{PO}$ CT GCA AC t tct	46.1 °C (-11.3)	55.8 °C (-0.8)

Table 1: Tm values[16] of the duplexes consisting of DNA, PNA-5'-DNA or PNA-5'-DNA-3'-PNA with the complementary DNA and RNA strands.

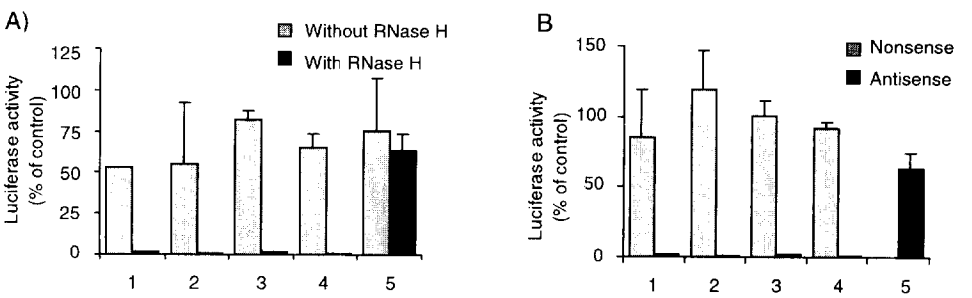


Figure 2: Effects of antisense oligos (1 μM) on the translation of the Luciferase gene. **Key**: Lane 1 = DNA, Lane 2 = DNA-(3')-PNA, Lane 3 = PNA-(5')-DNA **1a**, Lane 4 = PNA-(5')-DNA **1c**, Lane 5 = PNA.

At this stage, we were interested to find out whether PNA/DNA chimerae would exhibit antisense (AS) activity. To this end, the translation inhibitory effect of DNA-(3')-PNA, PNA-(5')-DNA **1a,c** and also PNA on Luciferase activity was investigated *in vitro* in rabbit reticulocyte lysate extracts (see Figure 2). The RNase H activity in this extract is known to be low under the conditions used for the *in vitro* translation.[17] It was established (see Figure 2A) that PNA/DNA chimerae targeting the initiation region of the mRNA had little effect on the translation of the Luciferase gene. Interestingly, addition of exogenous RNase H (*E. coli*) led to an efficient blocking of the translation process.[18] In this respect, it is important to note that addition of RNase H had hardly any effect on the inhibition activity of PNA, indicating that steric blocking alone (T_m>80°C) is not very effective. As expected, no significant inhibition was observed when oligos with nonsense (NS) sequences were used (see Figure 2B). These results imply that blocking of gene expression using PNA/DNA chimerae can be ascribed to RNase H dependent antisense effects.

In summary, a general and reliable method for the automated synthesis of PNA/DNA chimerae with different 5'-linkers is presented. The binding affinity of PNA-(5')-DNA and PNA-(5')-DNA-(3')-PNA chimerae with complementary DNA is lower than with native DNA. In contrast, PNA-(5')-DNA forms more stable duplexes with complementary RNA than DNA. It is also evident that the phosphonate bond $\mathbf{t^{PO}}$ has a minimal effect on duplex stability. Finally, antisense PNA/DNA chimerae are promising tools to inhibit translation in an RNase H assisted manner.

Experimental section

For the *in vitro* inhibition translation of the Luciferase gene the following oligos were used: Lane 1: NS DNA: GAA CTT CGA GAG TTA CCT, AS DNA: GTT TTT GGC GTC TTC CAT (T_m (RNA) = 62.4°C); Lane 2: AS DNA-(3')-PNA: GTT TTT GGC GTC T **t*** cca t (T_m (RNA) = 59.4°C); Lane 3: AS PNA-(5')-DNA **1a**: gtt tt ^{NH}**T** GGC GTC TTC CAT (T_m (RNA) = 60.8°C); Lane 4: AS PNA-(5')-DNA **1c**: gtt t $\mathbf{t^{PO}}$ TGG CGT CTT CCA T (T_m (RNA) 60.5°C); Lane 5: AS PNA: gtt ttt ggc gtc ttc cat (T_m (RNA) > 80°C). Translation was performed using a TNT® T7 coupled rabbit reticulocyte (RR) lysate system (Promega). The assay conditions were: 2.5 µL TNT RR lysate, 0.2 µL reaction buffer, 0.1 µL T7 Polymerase, 0.1 µL amino acid mixture without methionine, 0.1 µL amino acid mixture without leucine (1 mM each), 0.1 µL RNasein, 0.125 µL T7 Luciferase control DNA in a total volume of 4 µL. The AS (or NS) oligo (5 µM) was added (1 µL) and the reaction mixture was incubated for 90 min at 30 °C. In the experiments with exogenous RNaseH, 0.25 µL of this enzyme (GIBCO BRL, 2 units/µL) was added to the reaction mixture. After the incubation, the reaction was diluted with phosphate buffered Saline (PBS, 245 µL). 2.5 µL of this mixture was added to the Luciferase Assay Reagent (20 µL). The Luciferase activity was measured using a LUMAC Biocounter M1500 luminometer. All experiments were performed in duplo.

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9 The synthesis of building block **3** will be described in due course.
10 It is important to note that phosphonylation of MMT protected ethylenediamine with diphenyl phosphite led to an unseparable mixture of phosphonate mono- and diesters (as judged by ^{31}P NMR), probably due to partial cleavage of the MMT group.
11 Van der Laan, A.C.; Strömberg, R.; Van Boom, J.H.; Kuyl-Yeheskiely, E.; Efimov, V.A.; Chakhmakhcheva, O.G. *Tetrahedron Lett.*, 1996, *37*, 7857-7860 and references cited therein.
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14 See also Bergmann, F.; Bannwarth, W.; Tam, S.*Tetrahedron Lett.*, 1995, *36*, 6823-6826.
15 Abbreviations: Capital and small letters stand for DNA and PNA, respectively, a 3'-phosphate linkage is presented by the symbol t* (see ref. 6).
16 Tm values were measured in 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA buffer (pH = 7.0), at a concentration of 3 µM of each strand, by heating from 25°C to 85°C at 0.5 °C/min and measuring the UV absorbance at 260 nm
17 Cazenave, C.; Frank, P.; Büsen, W. *Biochimie*, 1993, *75*, 113-122.
18 It was found that the Luciferase gene was almost completely blocked at a PNA-(5')-DNA (*i.e.* 1a,c) concentration of 200 nM (data not shown).