A Novel *N*-(Pyrrolidinyl-2-methyl)glycine-Based PNA with a Strong Preference for RNA over DNA

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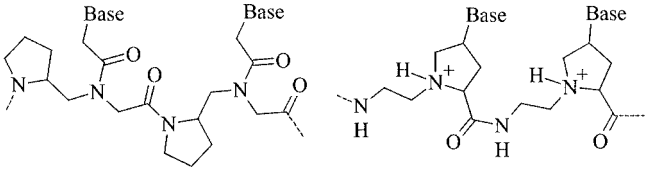
A novel, *N*-(pyrrolidinyl-2-methyl)glycine-based (Pmg-based) PNA is introduced. The synthesis of the backbone was accomplished in good yield, starting from prolinol. Thymine (*S*)- and (*R*)-Pmg and adenine- and cytosine-derived (*R*)-Pmg monomers were prepared. Five different fragments – two with either the (*R*) or the (*S*) isomer of the thymine Pmg monomer, two oligomers with two consecutive (*R*)- or (*S*)-thymine Pmg units, and fully modified (*R*)-Pmg decamer – were assembled on a solid support. UV thermal melting experiments with complementary DNA and RNA were performed in order to determine the effects of conformational restriction, steric hindrance, and chirality on the duplex stability. It was found that the (*R*)-Pmg-containing PNAs bound better to DNA and RNA than those containing the (*S*)-Pmg isomer and that both (*S*)- and (*R*)-Pmg-containing PNAs preferentially bound to complementary RNA with a selectivity higher than that of 2-(aminoethyl)glycine (Aeg) PNA. However, even (*R*)-Pmg-containing oligomers showed poorer duplex stability than nonmodified Aeg-PNA, while no detectable binding either to the DNA or to the RNA was observed with the fully modified (*R*)-Pmg decamer.

Introduction

PNA, an artificial nucleic acid, the backbone of which is composed of repeating 2-(aminoethyl)glycine (Aeg) units, forms stable complexes with complementary DNA and RNA.[1–5] Previous publications have shown that PNAs based on amino acids other than glycine possess similar properties.[6–10] These reports have demonstrated that the chirality of the backbone is an important factor determining the stability of the complexes. In addition, recent studies have indicated that the introduction of conformational restriction into oligonucleotides,[11,12] PNA-DNA[13,14] chimeras, and modified PNAs[15,16] has a beneficial effect on their hybridization properties. It is believed that suitable conformational restriction in the nucleic acid backbone should result in better positioning relative to the target strand, thus giving higher affinity. We were interested in investigating the hybridization properties of an uncharged PNA modification that would combine both conformation restriction and chirality, and so we designed and prepared a new chiral PNA analogue, the backbone of which contained both isomers of *N*-(2-pyrrolidinylmethyl)glycine (Pmg) **I**.

We reasoned that the pyrrolidine moiety would introduce restraints and chirality, while the backbone would still contain the requisite six covalent bonds in the monomeric unit, as in Aeg-PNA. Fragments would be constructed by introduction of an amide bond between the carboxyl and the pyrrolidineamino functions of two monomers. The second amino group in the Pmg monomeric unit would be the attachment point (carbonyl methylene) of the nucleobase. Unlike aminoethylprolyl PNA[17] (Aep-PNA, **II**) and pyrrolidine PNA[18] (not shown), which are positively charged (Figure 1), the Pmg backbone should remain uncharged at physiological pH. Polycationic species are generally known to form stable complexes with DNA or RNA due to charge attraction; however, nonspecific binding may be a consequence.[19]



Pmg-PNA, **I** Aep-PNA, **II**

Figure 1. Structures of Pmg- and Aep-PNAs

Study of the hybridization of Pmg-PNA should permit the determination of the conformation restriction and chirality effects on duplex formation in the absence of positive charge. This may provide useful information for the development of new PNA analogues.

As a part of an ongoing research program geared to the design, synthesis, and investigation of potential antisense agents and tools for biological studies,[20,21] we give here a

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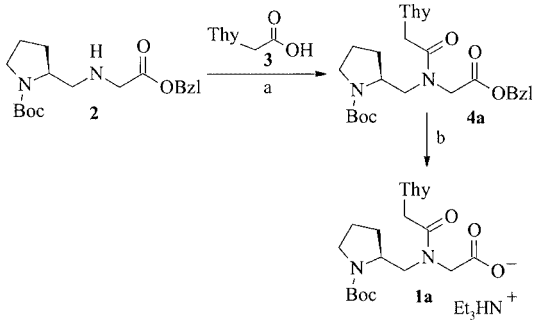
full report of the synthesis of and UV melting experiments involving Pmg-containing PNAs.

Results and Discussion

The first step in the assembly of Pmg-containing PNA fragments on a solid support was the preparation of properly protected Pmg building blocks. Initially we chose *tert*-butoxycarbonyl (Boc) as a temporary protecting group for the pyrrolidine amino group, and benzoyl for the exocyclic amino functions of the nucleobases.

Thymine (*S*)-Pmg unit **1a** was synthesized from commercially available benzyl *N*-[(2*S*)-*N*-Boc-pyrrolidinylmethyl]-glycinate[22] (**2**) (Scheme 1). Acylation of compound **2** at the free secondary amino function was performed with thymine-1-acetic acid (**3**) and the coupling agent 2-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of *N*-ethyldiisopropylamine (DIEA) and 4-(dimethylamino)pyridine (DMAP). The isolated yield of the fully protected (*S*)-Pmg monomer **4a** was 80%. Basic hydrolysis of the benzyl ester **4a** with aqueous NaOH and transformation of the sodium salt into triethylammonium salt yielded monomer **1a** in a form suitable for solid-phase synthesis.

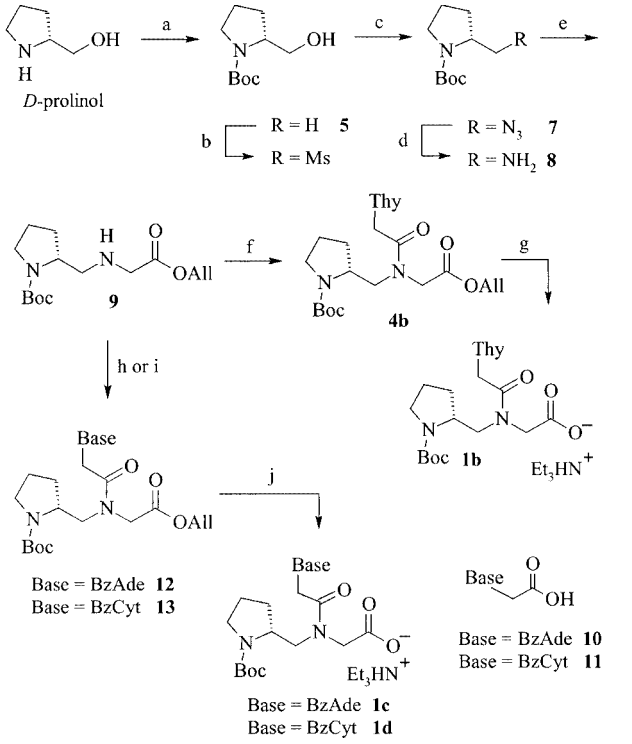


Scheme 1. Synthesis of thymine (*S*)-Pmg monomer; reagents: (a) HBTU, DIEA, DMF, DMAP; (b) 1) 2 M NaOH, dioxane, 2) $KHSO_4$ (pH 2.0), 3) Et_3N

Unlike the (*S*) isomer, (*R*)-Pmg was not commercially available, and so was prepared by the following route, starting from D-prolinol (Scheme 2).

The amino function of D-prolinol was protected with the Boc group and the primary alcohol **5** was then mesylated. Nucleophilic substitution of the mesylate **6** with lithium azide and subsequent reduction of azide **7** with triphenylphosphane in the presence of water afforded amine **8** in an overall yield of 84% from D-prolinol.[23]

Alkylation of amine **8** with allyl bromoacetate in the presence of triethylamine in THF gave the Boc-(*R*)-Pmg unit **9** in 66% isolated yield. Coupling of backbone **9** variously with thymine-1-acetic acid (**3**), N^6-benzoyladenine-9-acetic acid (**10**), or N^4-benzoylcytosine-1-acetic acid (**11**) afforded the completely protected (*R*)-Pmg building blocks **4b**, **12**, and **13**, respectively. Basic hydrolysis of ester **4b** was performed as in the above synthesis of (*S*)-Pmg, yielding

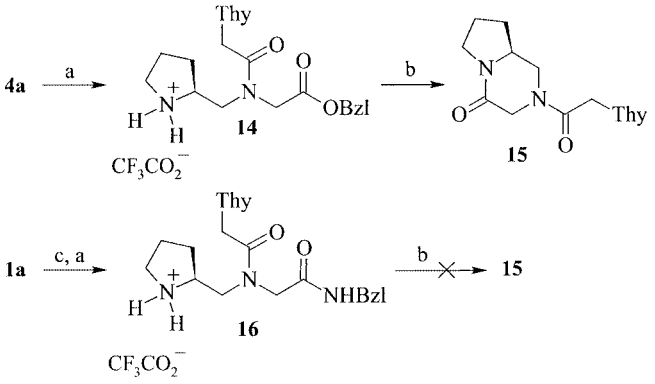


Scheme 2. Synthesis of adenine, cytosine and thymine (*R*)-Pmg monomers; reagents: (a) Boc_2O, 1 M NaOH (aq.), dioxane; (b) MsCl, Et_3N, THF; (c) LiN_3, DMF, 16 h, 64 °C; (d) Ph_3P, H_2O, THF; (e) $BrCH_2COOAllyl$, Et_3N, THF; (f) **3**, DIEA, HBTU, DMF; (g) 1) 2 M NaOH (aq.) dioxane, 2) $KHSO_4$ (pH 2), 3) Et_3N; (h) **10**, DIEA, HBTU, DMF; (i) **11**, DIEA, HBTU, DMF (j) Bu_3SnH, $[Pd(PPh_3)_2]Cl_2$, AcOH, CH_2Cl_2, 10 min

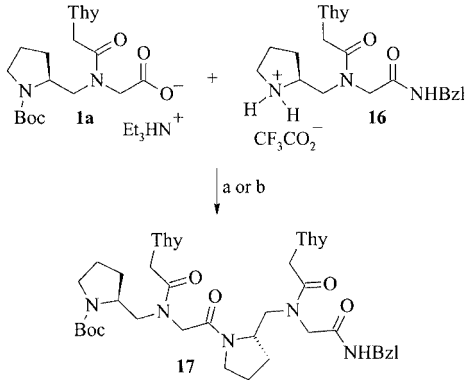
monomer **1b**. Units **1c** and **1d** were obtained by Pd-catalyzed deallylation[24] of compounds **12** and **13**, respectively.

It had been reported that dipeptides of the type H-Pro-Gly-OR were prone to cyclize under basic conditions.[25,26] In order to investigate whether and to what extent this undesired side-reaction would occur during the synthesis of Pmg-containing PNA fragments, the following experiment was set up. Compound **14** was produced by acidolysis of the fully protected thymine Boc-(*S*)-Pmg benzyl ester **4a** (Scheme 3). Such a system would be equivalent to a resin-bound thymine Pmg unit. This derivative was treated with a 0.4 M DIEA solution in DMF (the concentration and the amount were in accordance with the solid-phase PNA synthesis coupling cycle described in Table 1). Compound **14** was fully transformed within 20 minutes into a new, less polar (by TLC) compound. Data obtained from ^1H and ^{13}C NMR spectroscopy were consistent with the ketopiperazine **15**. The fast cyclization of compound **14** in the DIEA solution implied that attachment of the first Pmg-based unit to the solid support through an ester bond was not to be recommended. One way to circumvent the cyclization problem was to anchor the first Pmg monomer to the resin through an amide bond. Compound **16** was therefore prepared and exposed to the solution of DIEA in DMF as described above for compound **14**.

As expected, TLC and NMR analysis indicated no formation of compound **15** even after 20 hours.



Scheme 3. Reagents: (a) 50% TFA/CH_2Cl_2; (b) 15 equiv. DIEA as 0.4 M solution in DMF; (c) $BzlNH_2$, HBTU, DIEA, DMF



Scheme 4. Coupling in solution; reagents: (a) HBTU, DIEA, DMF; (b) PyBroP®, DIEA, DMF

Table 1. Steps involved in the solid phase synthesis by the mixed protection group strategy

Step	Function	Solvents and reagents[a]	Time [min]
1.	Fmoc deprotection of resin-bound glycine	22% piperidine in NMP (v/v)[b]	2 × 3.5
2.	Single coupling	MMT Aeg-PNA monomers[c], HBTU[c], DIEA[d]	17.5
3.	MMT deprotection during chain elongation	1% TFA in dichloroethane (v/v)[e]	3
4.	Double coupling	Boc Pmg-PNA monomer **1a** or **1b**[c], HBTU[c], DIEA[d] (double coupling)	2 × 17.5
5.	Boc deprotection	50% TFA in dichloromethane (v/v)[f]	2 × 15
6.	Lysine termination	Fmoc-L-Lys(Boc)-OH[c], HBTU[c], DIEA[d] (double coupling)	2 × 17.5

[a] Synthesis was performed on 41 mg support (1 μmol). [b] 3.5 mL. [c] 0.3 M solution in NMP. [d] 0.4 M solution in NMP. [e] 6 mL. [f] 10 mL.

This finding not only implied that linking of Pmg-based monomers to the resin through an amide bond would be advantageous over attachment through an ester bond but also that condensation of a chain-bound Pmg monomer should not result in any significant loss of the above building block as the result of cyclization.

Prior to the synthesis of the Pmg-containing PNA fragments on solid support, experiments were performed in order to identify suitable coupling conditions for the introduction of the amide bond between two Pmg units. Two coupling reagents − HBTU and bromotris(pyrrolidinyl)-phosphonium hexafluorophosphate (PyBroP®) − were selected for condensation of **1a** and **16** (Scheme 4). HBTU is commonly used both in solution and solid-phase peptide and PNA synthesis, while PyBroP® has been reported to give better yields for the coupling of hindered amino acids.[27,28]

In two parallel experiments, equal amounts of compounds **1a** and **16** in DMF were treated with 1.5 equivalents of the coupling agent, and the formation of dimer **17** was monitored by HPLC. In the case of HBTU, no further change in the composition of the reaction mixture could be observed after 55 min One major product was formed, and this was isolated (yield 93%) and characterized by ^1H and ^{13}C NMR spectroscopy and by mass spectrometry. It was identified as the desired dimer **17** (Scheme 4).

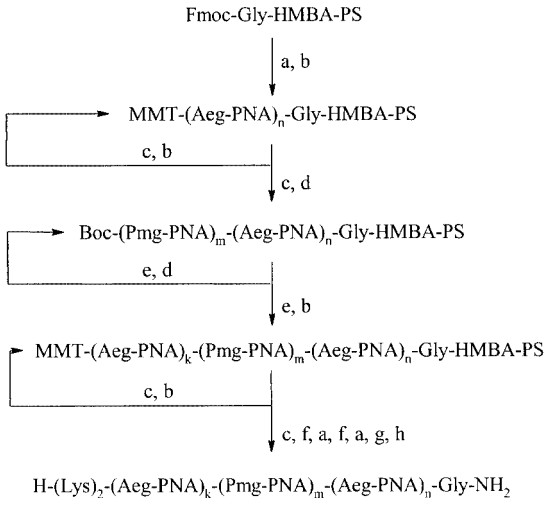
On the other hand, analysis of the PyBroP®-mediated coupling after 55 min showed two new compounds in a 1:4 ratio. The major of these was identified as the dimer **17**. No further progress of the reaction could be detected over 2.5 h. The minor product was possibly the symmetric anhydride of compound **1a**.

Initially, four PNA fragments containing one or two thymine (*S*)- or (*R*)-Pmg units in the middle of the strand (either at the fifth, or at both the fifth and the sixth position, counting from the *C*-terminal) were synthesized on a solid support by mixed protecting group strategies (Table 1 and Scheme 5). The solid support was preloaded with Fmoc-protected glycine, incorporated into the PNA strands to prevent ketopiperazine formation (as described above). The Aeg-PNA part was assembled with MMT/acyl-protected monomers.[29] The PNA chain was elongated by HBTU-mediated coupling of the appropriate monomers, followed by removal of the MMT group with 1% TFA in dichloroethane. The thymine Pmg building blocks **1a** or **1b** were incorporated under the same coupling conditions, executed twice prior to the acidolysis of the Boc group.

Thus, a 50% TFA solution in dichloromethane was manually applied to the disconnected synthesis vessel for the removal of the Boc group from monomers **1a** or **1b**.

In addition, to prevent acyl shift[30] and to increase the solubility, all fragments were terminated with two consecutive lysines.[1,31]

An essential point in the incorporation of the thymine Pmg units was the replacement of the resin neutralization step after Boc deprotection by an extensive wash. This change allowed the coupling yields of Pmg monomers to be improved from 71% to 99%. The average coupling yields of



Scheme 5. Solid-phase synthesis of Pmg-PNA by a mixed protecting group strategy; reagents: (a) 22% piperidine/NMP (v/v); (b) MMT-Aeg-PNA building block, DIEA, HBTU; (c) 1% TFA/dichloroethane (v/v); (d) Boc-Pmg-PNA building block **1a** or **1b**, DIEA, HBTU; (e) TFA/DCM 1:1 (v/v); (f) Fmoc-L-Lys(Boc)-OH, DIEA, HBTU; (g) 95% aq. TFA; (h) NH_3/MeOH saturated, 16 h, 55 °C

the Pmg building blocks were determined by comparison of the absorbance of the released MMT cation of the Aeg-PNA monomers coupled prior to and after the thymine Pmg unit(s). The assembled PNA fragments (Table 2, **IV**−**VII**) were detached from the solid support and the nucleobases deprotected by ammonolysis.[32] The oligomers were then purified by HPLC and analyzed by mass spectrometry.

The abilities of the Pmg-containing PNA fragments to form duplexes with complementary DNA and RNA were evaluated by UV thermal melting experiments. As reference points, the melting temperatures of PNA-DNA and PNA-RNA duplexes were used (Table 2, entries 3−4). PNA decamer **IV**, with a single thymine (*S*)-Pmg unit, gave T_m values of 21 °C and 39 °C when hybridized to complementary DNA and RNA, respectively (Table 2, entries 5, 6). Fragment **V**, containing two consecutive thymine (*S*)-Pmg units, showed no detectable transition with DNA and a T_m of 27 °C with target RNA (entries 9, 10). The above data for fragments **IV** and **V** imply a ΔT_m of 19 °C per single (*S*)-Pmg modification, calculated from the T_m of unmodified PNA duplex with DNA, and ΔT_m values of 14 °C and 13 °C for fragments **IV** and **V**, respectively, calculated from unmodified PNA duplex with RNA (see entries 5 vs. 3, 6 and 10 vs. 4).

PNA decamer **VI**, with a single thymine (*R*)-Pmg unit, gave T_m values of 24 °C and 44 °C when hybridized to complementary DNA and RNA, respectively (Table 2, entries 7, 8). Fragment **VII**, containing two consecutive thymine (*R*)-Pmg units, gave T_m values of 5 °C and 36 °C with complementary DNA and RNA, respectively (Table 2, entries 11, 12). The above data for fragments **VI** and **VII** imply a decrease of 17 °C per (*R*)-Pmg modification with DNA and 9 °C with RNA, relative to Aeg-PNA **III**.

In comparison with the oligomers containing the (*S*) isomer, the (*R*)-Pmg PNAs showed higher affinities for both DNA and RNA. The above T_m values also indicated that Pmg-PNAs (i.e., fragments **IV**−**VII**) bound complementary RNA with higher selectivity than Aeg-PNA. This effect was more pronounced with the (*R*)-Pmg-containing fragments **VI** and **VII**. For example, the ΔT_m values (RNA vs. DNA) were 20 °C and 31 °C for decamers **VI** and **VII**, respectively, compared to 13 °C for unmodified PNA **III**. The T_m values of decamer **VII** with DNA (entry 2, Table 2) and with complementary RNA were approximately the same (38 °C and 36 °C, respectively). The ability to bind RNA, however, was substantially higher, the ΔT_m (RNA vs. DNA) values being 3

Table 2. UV thermal melting data; all PNA sequences were of the following structure (N → C): H-$(Lys)_2$-$(PNA)_{10}$-Gly-NH_2; lowercase letters in PNA sequences denote Aeg-PNA (a, c, t), bold uppercase denote Pmg-PNA ($\mathbf{T^X}$) where the superscript letter indicates the isomer

Entry	PNA #	Sequences	T_m (°C)	ΔT_m(RNA vs. DNA) (°C)	ΔT_m/mod. (°C)
1.		d(TCACTTCCAT):DNA	35.0		
2.		d(TCACTTCCAT):RNA	38.0	3.0	
3.	**III**	tcacttccat:DNA	40.0		
4.	**III**	tcacttccat:RNA	53.0	13.0	
5.	**IV**	tcact$\mathbf{T^S}$ccat:DNA	21.0		−19.0[a]
6.	**IV**	tcact$\mathbf{T^S}$ccat:RNA	39.0	18.0	−14.0[b]
7.	**VI**	tcact$\mathbf{T^R}$ccat:DNA	24.0		−16.0[a]
8.	**VI**	tcact$\mathbf{T^R}$ccat:RNA	44.0	20.0	−9.0[b]
9.	**V**	tcac$\mathbf{T^S T^S}$ccat:DNA	−[c]		−[c]
10.	**V**	tcac$\mathbf{T^S T^S}$ccat:RNA	27.0	−	−13.0[b]
11.	**VII**	tcac$\mathbf{T^R T^R}$ccat:DNA	5.0		−17.5[a]
12.	**VII**	tcac$\mathbf{T^R T^R}$ccat:RNA	36.0	31.0	−8.5[b]
13.	**VIII**	(**TCACTTCCAT**)^R:DNA	−[c]		−[c]
14.	**VIII**	(**TCACTTCCAT**)^R:RNA	−[c]	−	−[c]

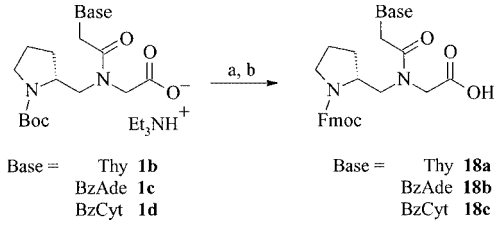
[a] Compared to the T_m of PNA:DNA duplex. [b] Compared to the T_m of PNA:RNA duplex. [c] No sigmoidal transition was observed.

°C and 31 °C for the RNA and compound **VII**, respectively.

All UV thermal melting experiments were performed at least three times and the deviation of the measured T_m values did not exceed 0.5 °C.

It had previously been reported that incorporation of modifications (single or multiple) in the nucleic acid strand could cause severe distortion of the duplex structure.[18] This so-called chimeric effect can be avoided by examining the hybridization of fully modified sequences.

Since (*R*)-Pmg-containing PNA fragments hybridized to DNA and RNA better than those containing the (*S*)-Pmg, a fully modified (*R*)-Pmg decamer **VIII** was assembled. In order to be able to perform a fully automated synthesis of fragment **VIII**, we followed the Fmoc strategy with building blocks **18a−c**, prepared from the previously synthesized Boc-monomers **1b−d** (See Scheme 6). Acidolysis of the Boc groups from compounds **1b−d** and subsequent treatment of the resulting intermediates with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocOSu) and DIEA in THF provided the desired building blocks **18a−c** in excellent yields after workup and purification.



Scheme 6. Synthesis of Fmoc (*R*)-Pmg monomers; reagents: (a) TFA/DCM/MeOH/H_2O 16:16:1:1 (v/v/v/v); (b) FmocOSu, DIEA, THF

Next, since the exocyclic amino groups of the adenine and cytosine nucleobases were protected with benzoyl groups, the stability of this protecting group in 22% piperidine solution in NMP was tested by TLC experiments on model compounds **19** and **20** (Figure 2). No cleavage products were detected in either case, and the starting compounds were intact after 3.5 h.

With the Fmoc (*R*)-Pmg monomers available and the nucleobase protecting group compatibility tested, an automated solid-phase synthesis of the fully modified (*R*)-Pmg-PNA decamer **VIII** was performed. Chain elongation and Fmoc deprotection were performed as described in Table 3 and Scheme 7.

All-Pmg-PNA fragment **VIII** was assembled starting from resin-linked glycine and terminated with two lysines. Detachment from the solid support and nucleobase protecting group cleavage afforded crude all-(*R*)-Pmg PNA, which was further purified by RP-HPLC and characterized by mass spectrometry.

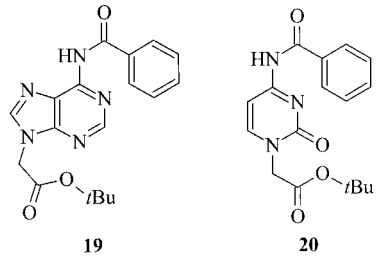
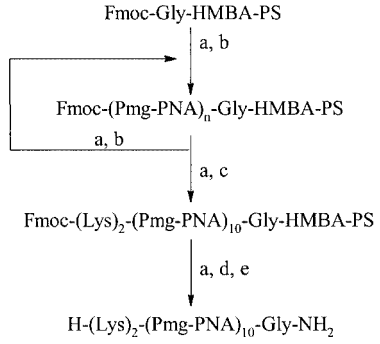


Figure 2. Model compounds for testing the stability of the *N*-benzoyl group in 22% piperidine



Scheme 7. Synthesis of all-(*R*)-Pmg PNA decamer **IX**; reagents: (a) 22% piperidine/NMP (v/v); (b) Fmoc-Pmg-PNA building block **18a−c**, DIEA, HBTU, 2×17.5 min; (c) Fmoc-L-Lys(Boc)-OH, DIEA, HBTU, 2×17.5 min; (d) 95% aqueous TFA; (e) NH_3/MeOH saturated, 16 h, 55 °C

Compound **VIII** was then subjected to the same UV thermal melting experiments as described above for the oligomers **III−VII**. Unfortunately, no thermal melting transition was observed either with complementary RNA or DNA. This inability to form stable duplexes may be due to unsuitable positioning of the nucleobases in the fully modified PNA strand **VIII**.

Conclusion

Both thymine (*S*)- and (*R*)-Pmg building blocks were synthesized in good yields. Four PNA decamers containing one or two thymine (*S*)- or (*R*)-Pmg units were assembled and their hybridization properties determined. The duplexes formed by (*R*)-Pmg-containing PNAs both with DNA and with RNA were more stable than those formed by thymine

Table 3. Steps involved in solid-phase synthesis of PNA **VIII**

Step[a]	Function	Solvents and reagents	Time (min)
1.	Fmoc deprotection	22% piperidine in NMP (v/v)[b]	2 × 3.5
2.	Coupling	Fmoc Pmg-PNA monomers **18a−c**[c], HBTU[c], DIEA[d], (double coupling)	2 × 17.5
3.	Lysine termination	Fmoc-L-Lys(Boc)-OH[c], HBTU[c], DIEA[d], (double coupling)	2 × 17.5

[a] Synthesis was performed on 41 mg support (1 μmol). [b] 3.5 mL. [c] 0.3 M solution in NMP. [d] 0.4 M solution in NMP.

(*S*)-Pmg-containing PNAs, but less stable than those obtained with unmodified Aeg-PNA. Both the (*R*)- and the (*S*)-Pmg-containing PNAs formed tighter duplexes with complementary RNA than with counterpart DNA. In addition, both the (*S*)- and the (*R*)-Pmg PNA exhibited substantially stronger preferences for RNA, compared to all-Aeg-PNA and native DNA. This enhanced selectivity, especially of the (*R*)-Pmg-containing fragments, towards RNA could be utilized in several possible ways. For example, a more stable RNA-preferring nucleic acid analogue might be created by increasing the Aeg-PNA population around (*R*)-Pmg based units. This analogue might be used to label or identify specific RNA in cell extracts, or gels and might have other applications in studies in which discrimination between RNA over DNA was desired.

Experimental Section

General Remarks: Solvents were purchased from Merck Eurolab. Dry solvents were obtained by use of appropriate molecular sieves, except for THF. The water content in dry solvents did not exceed 50 ppm as determined by Karl−Fisher titration. THF was distilled from sodium benzophenone ketyl. D-Prolinol was purchased from Aldrich. HBTU and PyBroP® were purchased from Novabiochem. TLC was performed on analytical Merck silica plates with F_{254} indicator. TLCs were viewed either under 254 nm UV or by staining with *o*-toluidine reagent or ninhydrin where appropriate. Solid-phase syntheses were performed on Pharmacia Gene Assembler Special with custom-written cycles. NMP for solid-phase synthesis was purchased from Applied Biosystems and dried over 4 Å molecular sieves. The solid phase was a low-loading (24.4 μmol/g), highly cross-linked polystyrene, loaded with Fmoc-glycine through a 4-(hydroxymethyl)-benzoic acid linker. Reversed-phase HPLC was performed on a Jasco HPLC system equipped with a Hypersil ODS 5 μm, 150 × 4.6 mm (Column 1) or a Phenomenex Jupiter 5 μm, 250 × 4.60 (Column 2) or 5 μm, 250 × 10.0 mm (Column 3). Buffer A was 0.1% TFA in water (pH 2.0), buffer B 90% aqueous acetonitrile with 0.1% TFA. The following gradients were used. Gradient A: 0−40% buffer B in 40 min; gradient B: 0−25% buffer B in 40 min 25−40% B in 20 min; gradient C: 0−50% buffer B in 50 min All runs were performed at 60 °C. Fractions containing the PNA were lyophilized. Pure Pmg PNAs were stored as frozen (−78 °C) solutions in deionized water. Complementary DNA was purchased from TAG Copenhagen A/S (Denmark), RNA from Dharmacon Research Inc. (USA). DNA and RNA sequences were purified by both cation exchange and reversed-phase HPLC, yielding chromatographically pure materials. Nominal mass spectra were recorded on Lasermat 2000 (Finnigan MAT) MALDI TOF (as a service at the Protein Analysis Centrum, Karolinska Institute) and/or Micromass LCT mass spectrometer equipped with an ES probe. Accurate masses were obtained on Micromass LCT mass spectrometer in positive ion mode with leucine enkephalin as an internal lock mass standard. NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer at 400.13 and 100.61 MHz for ^{1}H and ^{13}C, respectively. Thermal melting experiments were performed on a Varian Cary 300 spectrophotometer with programmable temperature block.

***N*-Boc-(*S*)-Pmg Thymine Monomer 1a:** DIEA (1.23 mL, 7.07 mmol) and thymine-1-acetic acid (**3**) (0.44 g, 2.36 mmol) were added to a solution of amine **2** (0.55 g, 1.57 mmol) in dry DMF (5.1 mL), followed by the addition of HBTU (0.90 g, 2.36 mmol). Shortly after the addition of HBTU, a precipitate formed. DMAP (0.29 g, 2.36 mmol) was added, and the reaction mixture was allowed to react until a clear solution was obtained (48 h room temperature), at which point TLC analysis indicated complete conversion of starting materials. The solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$, water, and acetate buffer (pH 4.0). The organic layer was then dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Crude product **4a** was purified by flash chromatography (FC) with ethyl acetate as eluent. Fractions containing the product were pooled and concentrated under reduced pressure. Ester **4a** was obtained as a white foam, in a yield of 81.0% (0.98 g). The benzyl ester **4a** (0.90 g, 1.75 mmol) was next dissolved in dioxane (5 mL) and water (1 mL) and hydrolyzed with 3 mL of 2 M aqueous NaOH. After 10 min the reaction mixture was neutralized to pH 7 (aq. $KHSO_4$), and the dioxane was removed under reduced pressure. The residue was diluted with ethyl acetate (25 mL), acidified to pH 2 (aq. $KHSO_4$), and extracted. The organic phase was washed with water, dried over Na_2SO_4, and concentrated. The obtained intermediate carboxylic acid was further converted into its triethylammonium salt by redissolving it in ethyl acetate and adding triethylamine, followed by FC purification (gradient 5 to 10% MeOH/CH_2Cl_2 + 1% Et_3N). Fractions containing the product were pooled, concentrated to dryness, co-evaporated with 5 mL CH_2Cl_2, and briefly dried in vacuo. The residue was redissolved in CH_2Cl_2, triturated from dry diethyl ether, and dried in vacuo. Compound **1a** was obtained as a white powder (0.77 g, 82.1%). ^{1}H NMR ($CDCl_3$, selected signals): δ = 9.49 (br. s, 1 H), 7.08 + 7.06 (s, 1 H), 4.85−4.25 (m, 3 H), 4.10−3.90 (m, 3 H), 3.90−3.70 (m, 1 H), 3.40−3.13 (m, 3 H), 3.07 (q, *J* = 7.3 Hz, 6 H), 1.85 (s, 3 H), 1.80−1.62 (br. s, 2 H), 1.44 + 1.42 (s, 9 H), 1.26 (t, 9 H, *J* = 7.3 Hz) ppm. ^{13}C NMR: δ = 173.5, 168.5, 165.0, 155.2 + 154.7, 151.7, 142.1, 110.6, 80.0 + 79.6, 56.0 + 55.7, 51.4, 49.9, 48.3, 47.0 + 46.6, 45.8, 29.4, 23.9 + 23.0, 12.8, 8.9 ppm. MS: *m*/*z* = 447.25 [M − Et_3N + Na]$^+$. Accurate mass calculated for $C_{19}H_{28}N_4NaO_7$ *m*/*z* = 447.1856, observed 447.1873.

(2*R*)-*N*-Boc-Pyrrolidinylmethanol (5): D-Prolinol (1.05 g, 10.4 mmol) was dissolved in a mixture of dioxane, water, and 2 M aqueous NaOH (20.8 mL, 15.6 mL, and 5.2 mL, respectively). The solution was cooled in an ice/water bath for 15 min, followed by addition of di-*tert*-butyl dicarbonate (Boc_2O, 2.5 g, 11.4 mmol). After the reagent had dissolved, the ice bath was removed and the reaction was allowed to proceed at ambient temperature until the starting material was consumed (1 h). The organic solvent was removed under reduced pressure, and ethyl acetate (50 mL) was added to the crude compound **5** in water. The pH was then adjusted to 7 and the title compound was extracted. The organic phase was washed with water (1 × 50 mL), dried with Na_2SO_4, and concentrated under reduced pressure, yielding 2.08 g (99.4%) of the compound **5** as a colorless oil, which solidified in vacuo overnight. The protected prolinol **5** was chromatographically pure (TLC, NMR) and was used for the next step without additional purification. ^{1}H NMR ($CDCl_3$): δ = 4.78 (br. s, 1 H), 3.95 (m, 1 H), 3.60 (m, 1 H), 3.42 (m, 1 H), 3.31 (m, 1 H), 1.99 (m, 1 H), 1.80 (m, 2 H), 1.55 (m, 1 H), 1.46 (s, 9 H) ppm. ^{13}C NMR: δ = 157.5, 80.6, 68.0, 65.3, 60.6, 47.9 + 47.2, 28.9, 24.5 ppm.

(2*R*)-2-Azidomethyl-*N*-Boc-pyrrolidine (7): Triethylamine (4.25 mL) was added to a solution of alcohol **5** (2.04 g, 10.1 mmol) in dry THF (103 mL), and the reaction flask was cooled in an ice/water bath for 15 min Mesyl chloride (1.56 mL, 20.2 mmol) was added in a single portion, resulting in instantaneous formation of a white

precipitate. After 1 h, the mixture was diluted with dichloromethane (120 mL), washed with saturated aq. $NaHCO_3$ (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The intermediate mesylate **6** was obtained as a colorless oil, and was used without further purification. Thus, mesylate **6** was dissolved in dry DMF (100 mL), and lithium azide (3.0 g, 60.6 mmol) was added. The reaction mixture was stirred and kept at 64 °C for 16 h, and then diluted with diethyl ether (250 mL), washed with water (1×500 mL, 3×250 mL) and saturated NaCl (50 mL), dried with $MgSO_4$, and concentrated under reduced pressure. Crude azide **7** was purified by silica FC, with isocratic eluent − *n*-hexane/ethyl acetate 3:2. Fractions containing the product were pooled and concentrated under reduced pressure, yielding azide **7** (1.93 g, 84.4%) as colorless liquid. ^{1}H NMR ($CDCl_3$): δ = 3.94 (m, 1 H), 3.58 + 3.37 (m, 4 H), 1.97 + 1.80 (m, 4 H), 1.47 (s, 9 H). ^{13}C δ = 154.9, 80.0, 56.9, 54.1 + 53.1 47.5 + 47.0, 28.9, 29.8 + 28.1, 24.3 + 23.4 ppm.

(2*R*)-2-Aminomethyl-*N*-Boc-pyrrolidine (8): Azide **7** (1.91 g, 8.44 mmol) in anhydrous THF (70 mL) was reduced with triphenylphosphane (4.43 g, 16.9 mmol) and water (0.312 mL, 17.3 mmol). The reaction mixture was heated to reflux until all the starting material had been consumed. The organic solvent was removed under reduced pressure. Diethyl ether (180 mL) was added to the remaining oil, and the pH of the aqueous phase was lowered to 1.75 with 2 M HCl with vigorous stirring. The aqueous layer was separated and washed with diethyl ether (2 × 90 mL), and the pH was then adjusted to 13.0 (2 M NaOH). After extraction with dichloromethane (6×100 mL), the combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure. Amine **8** was obtained in quantitative yield as a colorless liquid (1.71 g) and used without additional purification. ^{1}H NMR (MeOD): δ = 3.78 (m, 1 H), 3.33 (m, 2 H), 2.83 (dd, *J* = 12.7, 4.4 Hz, 1 H), 2.59 (m, 1 H), 1.87 (m, 4 H), 1.49 (s, 9 H) ppm. ^{13}C NMR: δ = 157.2 + 157.0, 81.4 + 81.1, 61.1, 48.5 + 48.1, 45.7, 29.6, 29.2 + 29.0, 24.9 + 24.2 ppm.

Allyl [*N*-(2*R*)-Boc-Pyrrolidin-2-yl)methyl]glycinate (9): Amine **8** (1.69 g, 8.44 mmol) was dissolved in a mixture of dry THF (45 mL) and triethylamine (1.18 mL, 8.44 mmol) and alkylated with allyl bromoacetate (1.51 g, 8.44 mmol) at ambient temperature. The reaction was allowed to continue overnight. The formed triethylammonium salt was removed by filtration, and the organic solvent was evaporated. The residue was then dissolved in ethyl acetate, washed with sat. $NaHCO_3$ and water, dried over Na_2SO_4, and concentrated. Pure compound **9** was obtained by silica FC with isocratic eluent − dichloromethane/ethyl acetate 1:1 + 0.2% Et_3N (v/v/v). The yield of compound **9** (colorless oil) was 65.8% (1.65 g). ^{1}H NMR ($CDCl_3$): δ = 5.91 (m, 1 H), 5.28 (dd, *J* = 10.3, 17.2 Hz, 2 H), 4.63 (d, *J* = 5.7 Hz, 2 H), 3.91 + 3.80 (m, 1 H), 3.46 + 3.32 (m, 4 H), 2.79 + 2.60 (m, 2 H), 1.94 + 1.80 (m, 4 H), 1.47 (br. s, 9 H) ppm. ^{13}C NMR: δ = 172.5, 155.3 + 155.0, 132.3, 119.0, 79.7, 65.7, 57.4, 53.0, 51.4, 47.2 + 46.8, 30.0 + 29.4, 28.9, 24.2 + 23.4 ppm. Accurate mass calculated for $C_{15}H_{27}N_2O_4$ *m*/*z* = 299.1971, observed 299.1979.

***N*-Boc-(*R*)-Pmg Thymine Monomer 1b:** Compound **1b** was prepared from backbone **9** (0.211 g, 0.706 mmol) as described above for monomer **1a**. Ester **4b** (0.330 g, 80.5%) was obtained as a white foam. Ester **4b** (0.300 g, 0.646 mmol) was then subjected to base hydrolysis as described above for compound **1a**, yielding **1b** as a white powder (0.286 g, 84.2%). ^{1}H NMR ($CDCl_3$): δ = 9.64 + 9.45 (s, 1 H), 7.10 (s, 1 H), 3.83 (m, 1 H), 3.10 (m, 3 H), 1.45 (s, 9 H) ppm. ^{13}C NMR: δ = 172.7 + 172.3, 168.1, 164.6, 154.9, 151.3, 141.7, 110.3, 79.7 + 79.3, 55.6 + 55.3, 51.0 + 50.0, 47.9, 46.7, 46.3 + 45.4, 28.5, 28.8, 23.5 + 22.6, 12.3, 8.5 ppm. MS: *m*/*z* = 526.25 $[M + H]^+$, 424.14 $[M - Et_3N + H]^+$. Accurate mass calculated for $C_{19}H_{28}N_4O_7Na$ *m*/*z* = 447.1856, observed 447.1851.

***N*-Boc-(*R*)-Pmg Adenine Monomer 1c:** Compound **12** was prepared as described above for compound **4a**, starting from backbone **9** with the slight modifications that DMAP was not used and that HBTU and N^6-benzoyladenine-9 acetic acid were 1.1 equivalents relative to backbone **9**. Ester **12** was obtained as a white foam (0.272 g, 56.4%). Pd-assisted deallylation proceeded as follows: ester **12** (0.257 g, 0.465 mmol) in dry dichloromethane (3.5 mL) was treated with Bu_3SnH (185 μL, 0.7 mmol) and $[Pd(PPh_3)_2]Cl_2$ (10 mg, 0.014 mmol) in the presence of acetic acid (40 μL, 0.70 mmol). The solution was stirred for 10 min, followed by addition of Et_3N (174 μL, 1.25 mmol). The reaction solvents were evaporated to near dryness, and the residue was redissolved in dichloromethane and precipitated from light petroleum ether. Crude product **1c** was purified by FC with a gradient of methanol in dichloromethane (0 to 10% MeOH) containing 1% Et_3N. Solvent removal under reduced pressure and trituration from dry diethyl ether yielded pure *N*-Boc-(*R*)-Pmg adenine **1c** as a white powder (0.253 g, 85.2%). ^{1}H NMR ($CDCl_3$): δ = 8.70 (d, *J* = 13.0 Hz, 1 H), 8.21 (d, *J* = 11.7 Hz, 1 H), 8.05 (dd, *J* = 1.4, 7.2 Hz, 2 H), 7.56 (m, 1 H), 7.48 (m, 2 H), 3.04 (q, *J* = 7.4 Hz, 6 H), 1.44 mi + 1.40 ma (s, 9 H), 1.25 (t, 9 H, *J* = 7.4 Hz) ppm. ^{13}C NMR (selected signals): δ = 173.4, 167.6, 165.4, 154.7, 152.7, 149.8, 145.3, 134.2, 133.1, 129.2, 128.4, 122.6, 79.6, 66.3, 55.7, 52.0, 50.2, 47.0, 45.6, 44.6, 34.6, 30.7, 30.1, 27.5, 23.9, 15.7, 9.0 ppm. Accurate mass calculated for $C_{26}H_{32}N_7O_6$ *m*/*z* = 538.2414 and $C_{26}H_{32}N_7NaO_6$ *m*/*z* = 560.2234, observed 538.2412 and 560.2221.

***N*-Boc-(*R*)-Pmg Cytosine Monomer 1d:** Compound **1d** was prepared as described above for compound **1c**, with backbone **9** (0.208 g, 0.697 mmol) and N^4-benzoylcytosine-1-acetic acid (0.220 g, 0.805 mmol). The yield of ester **13** was 0.147 g (36.2%). After the removal of the allyl ester, compound **1d** was obtained as a white power in a yield of 93.8% (0.135 g). ^{1}H NMR ($CDCl_3$, selected signals): δ = 7.96 (d, *J* = 7.4 Hz, 2 H), 7.75 (m, 1 H), 7.59 (m, 2 H), 7.50 (m, 2 H), 3.09 (m, 2 H), 1.44 (s, 9 H) ppm. ^{13}C NMR: δ = 173.1, 168.4, 167.3, 163.4, 156.2, 155.2, 151.4, 133.4, 129.2, 128.4, 128.3, 97.3, 79.8, 55.8, 53.0, 51.1, 47.1, 45.9, 29.3, 28.9, 23.9, 23.0, 8.9, 7.9 ppm. Accurate mass calculated for $C_{25}H_{32}N_5O_7$ *m*/*z* = 514.2302 and $C_{25}H_{31}N_5NaO_7$ *m*/*z* = 536.2121, observed 514.2291 and 536.2124.

***N*-Fmoc-(*R*)-Pmg Cytosine Monomer 18c:** Compound **1d** (0.535 g, 0.87 mmol) was dissolved in CH_2Cl_2 (8 mL), MeOH (0.5 mL), and H_2O (0.5 mL). TFA (8 mL) was added. Once TLC analysis indicated complete acidolysis of the Boc group, the mixture was diluted with toluene (20 mL) and concentrated under reduced pressure. The resulting oily residue was co-evaporated with toluene (2×20 mL) and then with dry THF (2×10 mL). The remaining yellow oil was dissolved in dry THF (7 mL), and DIEA (0.662 mL, 1.92 mmol) was added, resulting in the formation of a dense, white precipitate. Subsequently, dry DMF (1 mL) and FmocOSu (0.324 g, 0.96 mmol) were added. The reaction vessel was sealed and placed in an ultrasonic bath until a clear solution resulted. After 16 hours, TLC analysis indicated complete conversion of the starting material into the product. The solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (30 mL), washed with aq. $KHSO_4$ (1×10 mL, pH 1.46) and water (3×10 mL), dried over Na_2SO_4, and concentrated to yield a white foam. The product was then transformed into a fine powder by redissolving it in CH_2Cl_2 and triturating from *n*-hexane. Yield 0.493 g (89.2%). ^{1}H NMR ($CDCl_3$, selected signals): δ = 7.95 (d,

J = 7.2 Hz, 2 H), 7.86−7.73 (m, 3 H), 7.65−7.57 (m, 3 H), 7.57−7.46 (m, 3 H), 7.42−7.34 (m, 2 H), 7.33−7.25 (m, 2 H), 7.02−4.80 (m, 3 H), 4.77−4.52 (m, 2 H), 4.50−4.28 (m, 2 H), 4.11−3.74 (m, 1 H), 3.74−3.56 (m, 1 H), 2.10−1.55 (m, 4 H) ppm. ^{13}C NMR: δ = 171.3, 168.0, 164.1, 157.5, 156.1 + 155.9, 151.2, 144.3, 141.7, 133.6, 133.1, 128.8, 128.2, 127.9, 127.5, 127.2, 125.1, 124.8, 120.0, 97.4, 78.5, 78.3, 67.5, 66.4, 57.0 + 55.9, 50.6, 28.7 + 28.3, 23.6 + 22.4 ppm. MS: m/z = 636.61 $[M + H]^+$, 658.61 $[M + Na]^+$, 674.59 $[M + K]^+$. Accurate mass calculated for $C_{35}H_{34}N_5O_7$ m/z = 636.2458, observed 636.2477. $C_{35}H_{33}N_5O_7$ (635.6826): calcd. C 66.13, H 5.23, N 11.02; found C 65.91, H 5.32, N 10.94. The ^{1}H spectrum of the above compound **18c** was complex and poorly resolved. In order to obtain a better resolved spectrum, the Fmoc group of compound **18c** (11 mg) was removed by β-elimination with 22% piperidine solution in dry DMF (1 mL). After 7 minutes the pH of the solution was reduced to 2 (aqueous TFA). The deprotected compound was then purified by RP-HPLC (Column 3, gradient: 0−75% buffer B in 60 min at 30 °C). The appropriate fractions were pooled and lyophilized to yield the pure compound as a hygroscopic trifluoroacetate salt. ^{1}H NMR (D_2O): δ = 7.96 mi + 7.90 ma (d, J = 7.2 Hz, 1 H), 7.81−7.79 (m, 2 H), 7.61−7.57 (m, 1 H), 7.48−7.44 (m, 2 H), 7.38−7.35 (m, 1 H), 4.73 (AB, 2 H, J = 16.6), 4.26 (AB, 2 H, J = 19.2), 3.87 (dd, J = 9.6, 14.9 Hz, 1 H), 3.77 (ddd, J = 2.9, 9.5, 12.4 Hz, 1 H), 3.46 (dd, J = 3.1, 14.9 Hz, 1 H), 3.40−3.22 (m, 1 H), 3.22−3.13 (m, 1 H), 2.14−2.04 (m, 1 H), 2.03−1.83 (m, 2 H), 1.62 (ddt, J = 13.2, 8.9, 8.8 Hz, 1 H) ppm. ^{13}C NMR: δ = 173.3, 170.6, 170.2, 164.1, 157.6, 151.9, 134.0, 132.9, 129.3, 128.4, 98.9, 59.6, 51.9, 51.0, 50.0, 46.0, 45.7, 27.6, 22.6 ppm. Accurate mass of the deprotected monomer calculated for $C_{20}H_{24}N_5O_5$ m/z = 414.1777, observed 414.1776.

***N*-Fmoc-(*R*)-Pmg Thymine Monomer 18a:** Compound **18a** was prepared as described for **18c**, starting from **1b** (0.752 g, 1.43 mmol). Yield 0.692 g (88.5%) as a white powder. ^{1}H NMR ($CDCl_3$): δ = 10.15 + 10.10 (br. s, 1 H), 7.85−7.70 (m, 2 H), 7.70−7.50 (m, 2 H), 7.50−7.35 (m, 2 H), 7.35−7.20 (m, 2 H), 7.05−7.00 (s, 1 H), 4.85−4.55 (m, 1 H), 4.50−4.35 (m, 2 H), 4.35−4.15 (m, 2 H), 4.15−3.95 (m, 1 H), 3.85−3.60 (m, 1 H), 3.55−3.10 (m, 3 H), 1.83 + 1.82 (s, 3 H) ppm. ^{13}C NMR: δ = 171.8, 168.6, 165.6, 156.0, 151.8, 144.3 + 144.2, 142.3, 141.7, 128.2 + 127.5, 125.4 + 125.0, 120.4, 111.0, 73.3, 67.9 + 66.6, 57.3, 56.2, 51.5, 50.0 + 49.7, 49.0, 48.3, 47.6, 46.8, 29.3 + 28.8, 25.9, 24.1 + 23.9, 22.9, 12.6 ppm. MS: m/z = 547.57 $[M + H]^+$. Accurate mass calculated for $C_{29}H_{30}N_4O_7Na$ m/z = 569.2012, observed 569.2008. $C_{29}H_{30}N_4O_7$ (546.5851): calcd. C 63.73, H 5.53, N 10.25; found C 63.55, H 5.67, N 9.99. A Fmoc-deprotected sample for NMR was prepared as described above for compound **18c**. ^{1}H NMR (D_2O): δ = 7.29 + 7.24 (s, 1 H), 4.56 (AB, 2 H, J = 16.9), 4.26 (AB, 2 H, J = 20.2), 3.87 (dd, J = 15.0, 9.2 Hz, 1 H), 3.75−3.73 (m, 1 H), 3.35−3.22 (m, 1 H), 3.42 (dd, J = 15.0, 3.1 Hz, 1 H), 2.15−2.05 (m, 1 H), 2.02−1.82 (m, 2 H), 1.76 (s, 3 H), 1.61 (ddt, J = 13.1, 8.7, 8.8 Hz, 1 H) ppm. ^{13}C NMR: δ = 143.4, 55.7, 51.6, 50.3, 48.9, 47.1, 28.6, 23.8, 11.7 ppm. Accurate mass of deprotected monomer calculated for $C_{14}H_{21}N_4O_5$ m/z = 325.1512, observed 325.1523.

***N*-Fmoc-(*R*)-Pmg Adenine Monomer 18b:** Compound **18b** was prepared as described for **18c**, starting from **1c** (0.752 g, 1.43 mmol). Yield 95.0% as a white powder. ^{1}H NMR (MeOD, selected signals): δ = 7.46 (d, J = 8.0 Hz, 2 H), 7.23−7.08 (m, 2 H), 7.05−6.86 (m, 5 H), 6.82−6.58 (m, 4 H), 3.70−3.50 (m, 2 H), 3.26−3.00 (m, 1 H), 2.85−2.75 (m, 1 H), 2.65−2.50 (m, 1 H) ppm. ^{13}C NMR: δ = 171.3, 168.4, 167.0, 152.1, 149.7, 145.7, 144.2, 141.6, 134.0, 132.9, 128.8, 128.4, 127.9, 127.5, 127.2, 125.1, 122.8, 120.0, 78.5, 78.3, 67.4 + 66.5, 57.0 + 56.0, 51.2, 44.3, 28.8 + 28.3, 23.4 ppm. MS: m/z = 660.66 $[M + H]^+$, 682.70 $[M + Na]^+$, 698.64 $[M + K]^+$. Accurate mass calculated for $C_{36}H_{34}N_7O_6$ m/z = 660.2571, observed 660.2568. A Fmoc-deprotected sample for NMR was prepared as described above for compound **18c**. ^{1}H NMR (D_2O): δ = 8.79 (s, 1 H), 8.58 (s, 1 H), 8.00 (d, J = 7.2 Hz, 2 H), 7.70 (t, J = 7.5 Hz, 1 H), 7.58 (t, J = 7.7 Hz, 2 H), 5.40 (s, 2 H), 4.45 (AB, 2 H, J = 19.2), 3.98 (dd, J = 9.3, 14.8 Hz, 1 H), 3.87 (ddd, J = 12.5, 3.2, 9.7 Hz, 1 H), 3.57 (dd, J = 3.5, 14.9 Hz, 1 H), 3.39−3.21 (m, 2 H), 2.24−2.12 (m, 1 H), 2.11−1.94 (m, 2 H), 1.72 (ddt, J = 13.3, 8.9, 8.9 Hz, 1 H) ppm. ^{13}C NMR: δ = 173.3, 169.9, 150.1, 147.9, 147.1, 134.3, 132.2, 129.3, 128.7, 121.2, 59.5, 50.9, 49.9, 45.8, 45.7, 27.6, 22.5 ppm. Accurate mass of deprotected monomer calculated for $C_{21}H_{24}N_7O_4$ m/z = 438.1890, observed 438.1875.

Reference Aeg-PNA H-Lys-Lys-tcacttccat-Gly-NH_2 (III): Solid-phase synthesis was performed by a mixed Fmoc/MMT protecting group strategy on 41.0 mg (1 μmol scale) of nonswelling, highly cross-linked aminomethyl-polystyrene, preloaded with Fmoc-glycine attached through a 4-(hydroxymethyl)benzoic acid (HMBA) linker (loading: 24.4 μmol/g, as determined spectrophotometrically by the amount of released dibenzofulvene). The assembly of the PNA was performed after cleavage of the Fmoc group from resin-bound glycine with 22% piperidine in NMP. The procedure for one elongation/deprotection cycle consisted of (1) wash: NMP (5 mL); (2) coupling: MMT-Aeg-PNA monomer (15 equiv.), HBTU (16 equiv.), DIEA (32 equiv.) in NMP (550 μL), 17.5 min; (3) wash: NMP (5 mL); (4) MMT deprotection: 1% TFA/dichloroethane (6 mL), 3 min; (5) wash: dichloroethane (2.5 mL) 1 min The MMT-Aeg-PNA monomers and HBTU were delivered as 0.3 M solutions in NMP. DIEA was delivered as a 0.4 M solution. The PNA decamer was terminated with two consecutive L-lysines by use of commercially available Fmoc-L-Lys(Boc)-OH. The procedure for the lysine incorporation/deprotection cycle consisted of (1) wash: NMP (5 mL); (2) double coupling: lysine monomer (15 equiv.), HBTU (16 equiv.), DIEA (32 equiv.) in NMP (550 μL), 17.5 min NMP wash (2.5 mL), lysine monomer (15 equiv.), HBTU (16 equiv.), DIEA (32 equiv.) in NMP (550 μL), 17.5 min; (3) wash: NMP (5 mL); (4) Fmoc deprotection: 22% piperidine/NMP (3.5 mL), 3.5 min NMP (2.5 mL), 22% piperidine/NMP (3.5 mL), 3.5 min Prior to the cleavage from the solid support, the N^{ε}-Boc groups of the lysines were deprotected with 95% aqueous TFA, followed by neutralization with 10% DIEA/CH_2Cl_2, washing with additional CH_2Cl_2 and diethyl ether, and then drying in vacuo. Release of the PNA fragment from the support was achieved by ammonolysis of the HMBA linker with saturated anhydrous ammonia/methanol at 55 °C/16 hours. Crude PNA **III** was purified by reversed-phase HPLC. The appropriate fractions were pooled and lyophilized. MS (MALDI-TOF): calculated for $C_{120}H_{165}N_{56}O_{35}$ m/z = 2950.3, observed 2951.4 $[M + H]^+$, 2973.3 $[M + Na]^+$.

PNA-Containing one Thymine (*S*)-Pmg Unit (IV): The solid-phase synthesis of PNA **IV** was performed by a mixed Fmoc/MMT/Boc strategy. The Aeg part of decamer **IV** was assembled as described above for PNA **III**. Attachment of thymine (*S*)-Pmg monomer **1a** was executed as follows: the Pmg unit was double-coupled (i.e., after the deprotection of MMT group from the last Aeg-PNA unit, building block **1a** was attached by the same procedure as described for the elongation of the Aeg-part). In this case, however, after an additional washing step, the coupling cycle was repeated. Next, the synthesis vessel was disconnected from the synthesizer and the removal of the Boc group was accomplished manually by application of a TFA solution (TFA/DCM, 1:1 v/v, 2 × 5 mL, 2 × 15 min) into and through the vessel by syringe. The synthesis vessel was then reconnected to the synthesizer, on which the support-bound

oligomer was additionally washed with dichloroethane (4 mL), acetonitrile (2 mL), and NMP (2 mL). The automated synthesis of the remaining Aeg-PNA was then resumed and performed as described above. Comparison of the absorbance of the released MMT cation from the fourth and sixth Aeg PNA monomers (i.e., before and after the thymine (*S*)-Pmg unit) indicated an average coupling efficiency of 89.0%. Deprotection, cleavage from the resin, and purification was performed as described above for the synthesis of PNA **III** (R_t = 24.0 min Column 1, gradient A). MS (MALDI-TOF): calculated for $C_{123}H_{169}N_{56}O_{35}$ *m/z* = 2992.0, observed 2991.7 [M + H]$^+$.

PNA-Containing Two Thymine *S*-Pmg Units (V): Compound **V** was prepared as described above for compound **IV**, with the exception that two thymine (*S*)-Pmg units were incorporated. Average coupling efficiency for (*S*)-Pmg unit was 90.1% (R_t 23.8 min Column 1, gradient B). MS (MALDI-TOF): calculated for $C_{126}H_{173}N_{56}O_{35}$ *m/z* = 3030.4, observed 3032.5 [M + H]$^+$.

PNA-Containing One Thymine (*R*)-Pmg Unit (VI): Compound **VI** was prepared as described for PNA **IV**, with the exception that thymine (*R*)-Pmg monomer **1b** was used. Average coupling efficiency for the thymine (*R*)-Pmg unit was 72.0% (R_t 12.2 min column 2, gradient A). MS (MALDI-TOF): calculated for $C_{123}H_{169}N_{56}O_{35}$ *m/z* = 2992.0, observed 2992.2 [M + H]$^+$.

PNA-Containing Two Thymine (*R*)-Pmg Units (VII): Compound **VII** was prepared as described for PNA **IV**, with the exception that two thymine (*R*)-Pmg units were incorporated. Average coupling efficiency for the Pmg unit 98.8% (R_t 17.0 min Column 2, gradient B). MS (MALDI-TOF): calculated for $C_{126}H_{173}N_{56}O_{35}$ *m/z* = 3030.4, observed 3032.5 [M + H]$^+$, 3054.4 [M + Na]$^+$, 3070.4 [M + K]$^+$.

All (*R*)-Pmg PNA (VIII): Solid-phase synthesis was performed by the Fmoc strategy. The chain elongation/deprotection cycle was identical to that used for lysine incorporation and described above for the synthesis of PNA **III**, with the exception that monomers **18a**−**c** were used. Deprotection, cleavage from the resin, and purification were performed as described above for synthesis of PNA **III**. Average coupling efficiency could not be determined due to the setup of the synthesizer, which was MMT dedicated (R_t 27.2 min column 2, gradient C). MS (ES-TOF): calculated for $C_{150}H_{205}N_{56}O_{35}$ *m/z* = 3350.6, observed 1676.6 [M + 2 H]$^{2+}$, 1118.4 [M + 3 H]$^{3+}$, 838.9 [M + 4H]$^{4+}$.

Thermal Melting Experiments: Melting temperatures were measured with 1:1 molar mixtures of PNA and the corresponding target DNA or RNA (amounts determined by the UV absorption and ε_{260}), each at a concentration of 3 mM in 10 mM phosphate buffer, pH 7, containing 100 mM NaCl, and 0.1 mM EDTA. Prior to the measurement of the melting profiles, the solutions were heated to 90 °C for 5 minutes at a fast rate, then cooled to 0.5 °C over 90 min and equilibrated at this temperature for 45 min Subsequently, the absorbance A_{260} was recorded vs. temperature for both dissociation and annealing at the rates 0.5 °C/min and 0.2 °C/min, respectively.

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