Influence of the Type of Junction in DNA-3′-Peptide Nucleic Acid (PNA) Chimeras on Their Binding Affinity to DNA and RNA

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

The automated on-line synthesis of DNA-3′-PNA (PNA = Polyamide Nucleic Acids) chimeras **1**–**3** is described, in which the 3′-terminal part of the oligonucleotide is linked to the aminoterminal part of the PNA either *via* a *N*-(2-mercaptoethyl)- (X = S), a *N*-(2-hydroxyethyl)- (X = O), or a *N*-(2-aminoethyl)- (X = NH) *N*-[(thymin-1-yl)acetyl]glycine unit. Furthermore, the DNA-3′-PNA chimera **4** without a nucleobase at the linking unit was prepared. The binding affinities of all chimeras were directly compared by determining their T_m values in the duplex with complementary DNA, RNA, or DNA containing a mismatch or abasic site opposite to the linker unit. We found that all investigated chimeras with a nucleobase at the junction form more stable duplexes with complementary DNA and RNA than the corresponding unmodified DNA. The influence of X on duplex stabilization was determined to be in the order O > S ≈ NH, rendering the phosphodiester bridge the most favored linkage at the DNA/PNA junction. The observed strong duplex-destabilizing effects, when base mismatches or non-basic sites were introduced opposite to the nucleobase at the DNA/PNA junction, suggest that the base at the linking unit contributes significantly to duplex stabilization.

Introduction. – Peptide or Polyamide Nucleic Acids (PNAs) are nucleic acid mimetics, in which the entire sugar-phosphate backbone is replaced by non-ionic *N*-(2-aminoethyl)glycine units [1]. They bind to complementary DNA or RNA targets with higher affinity than natural oligonucleotides following the *Watson-Crick* rules of base pairing [2], and they are resistant to enzymatic degradation [3]. These properties make them attractive candidates for the use as antisense therapeutics and diagnostics [4]. Recently, we have shown that some limitations of PNAs, such as low cellular uptake, ambiguous orientation on hybridization (parallel or antiparallel), the inability to activate RNase H, and the propensity to self-aggregation, can be overcome by using DNA-PNA chimeras [5][6]. In these chimeras (see **1**), the 3′-part of the oligodeoxynucleotide was linked to a terminal (hydroxyethyl)glycine unit (X = O) of the PNA *via* a phosphodiester linkage [5][7]. For PNA-5′-DNA chimeras with the 5′-part of the DNA linked to the carboxy terminus of the PNA, a significant influence of the type of linkage at the DNA/PNA junction has been reported previously [8]. Here, we describe the synthesis and binding properties of novel DNA-3′-PNA chimeras containing all four nucleobases, which are linked by a *N*-(2-mercaptoethyl)glycine (**2**; X = S) or an *N*-(2-aminoethyl)-glycine (**3**; X = NH) [9] unit, respectively. To aid the optimal future design of DNA-3′-PNA chimeras, we directly compare the binding affinity of all three differently linked DNA-3′-PNA chimeras **1**–**3** in the duplex with complementary

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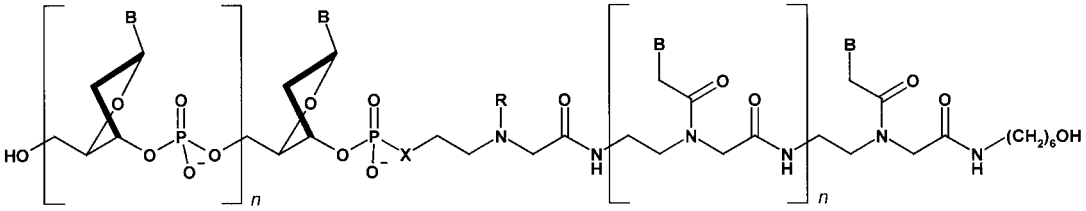
DNA and RNA. To evaluate the contribution of the linking moiety in these chimeras to their binding affinity, we have also prepared and investigated the DNA-3′-PNA chimera **4** with an abasic *N*-(2-hydroxyethyl)glycine unit at the DNA/PNA junction (R = H, X = O).



X = O, S, NH ; R = H , R^1 R^1 = Thy

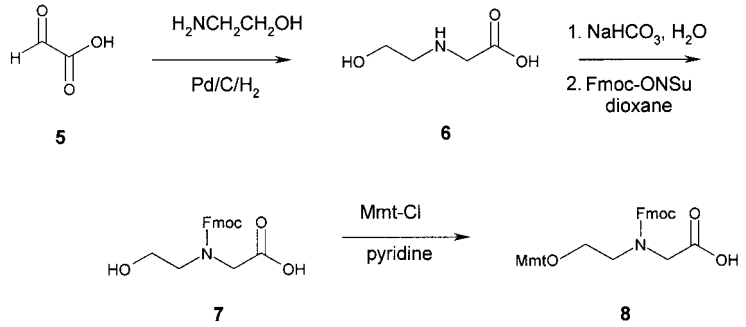
B = adenine, guanine, cytosine, or thymine

	X	R
1	O	R^1
2	S	R^1
3	NH	R^1
4	O	H

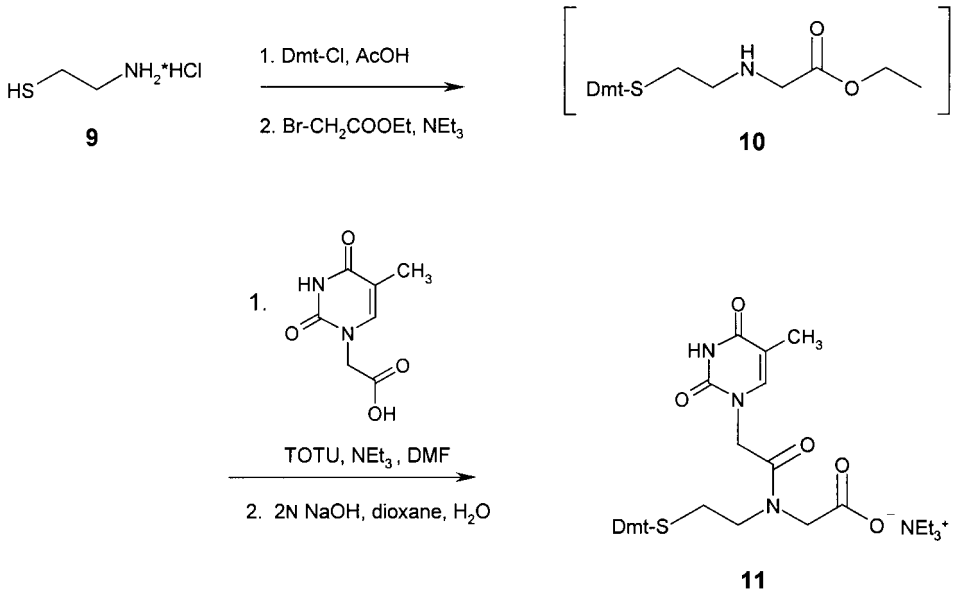
Results and Discussion. – *Synthesis of the Monomeric Linker Units.* For the incorporation of the abasic linker unit (X = O, R = H) at the DNA/PNA junction of the chimeras, we synthesized first the orthogonally protected monomeric linker unit **8** (*Scheme 1*). Reductive amination of 2-aminoethanol with glyoxylic acid in H_2O with H_2 as reducing agent and Pd/C as catalyst [10] resulted in *N*-(2-hydroxyethyl)glycine (**6**) in 83% yield. The amino function of **6** was then protected by reaction with (9*H*-fluoren-9-yl) methyl *N*-succinimidyl carbonate in dioxane to give the [(9*H*-fluoren-9-yl)methoxy]-carbonyl (Fmoc) derivative **7** in 77% yield. Finally, the abasic linker unit **8** was obtained in 70% yield by reaction of the OH group of **7** with (4-methoxyphenyl)diphenylmethyl (Mmt) chloride in pyridine.

The monomeric linker unit **11** (*Scheme 2*), which is based on *N*-(2-mercaptoethyl)glycine, was prepared from 2-mercaptoethylamine hydrochloride by reaction with bis(4-methoxyphenyl)phenylmethyl (Dmt) chloride and successive *N*-alkylation with ethyl 2-bromoacetate and NEt_3 in DMF to give *N*-[2-(Dmt-thio)ethyl]glycine ethyl ester (**10**). Intermediate **10** was coupled without further purification with thymine-3-

Scheme 1



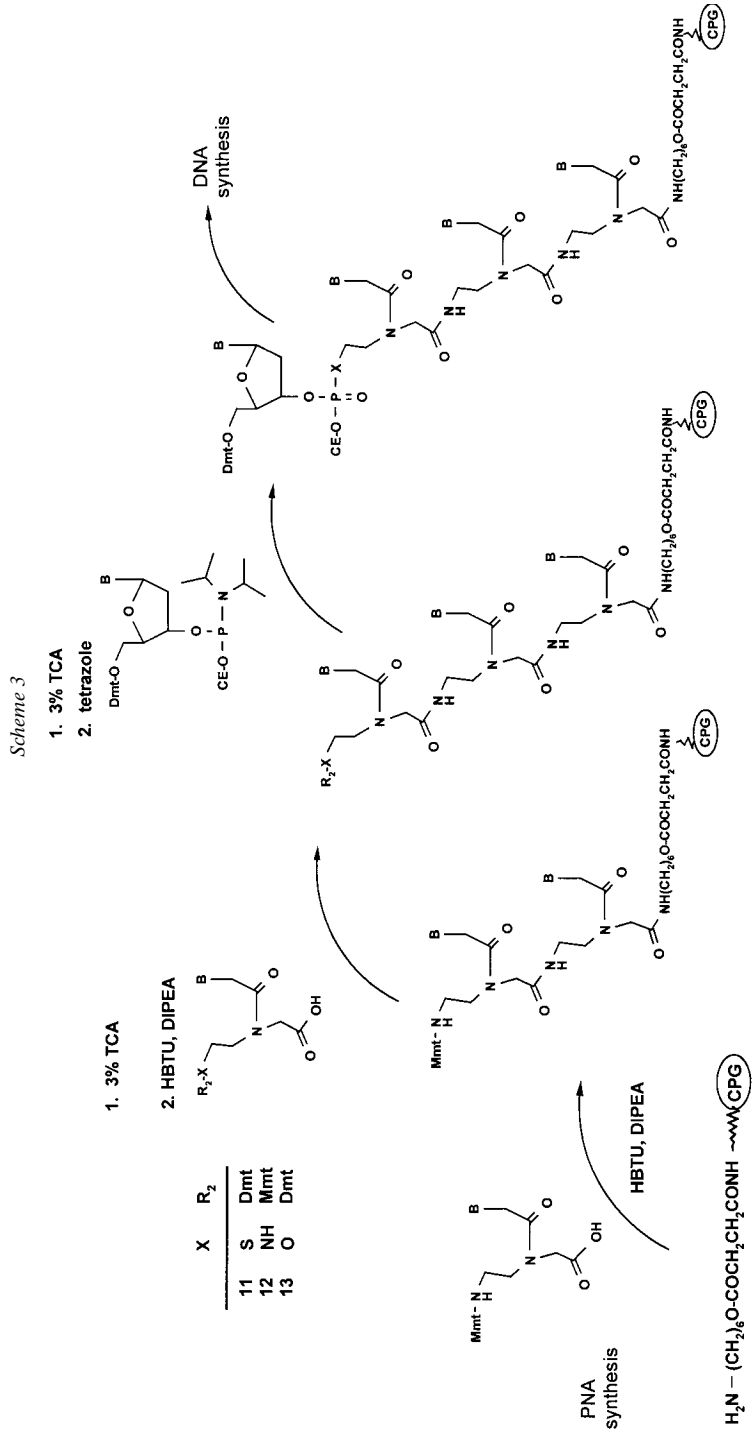
Scheme 2



acetic acid using *O*-{[(2-cyanoethoxycarbonyl)methylidene]amino}-1,1,3,3-tetramethyluronium tetrafluoroborate (TOTU) and then saponified with 2N NaOH in dioxane/H_2O 2:1 (*v*/*v*). After purification of the crude product by silica-gel chromatography with 1% NEt_3 in CH_2Cl_2/MeOH, the (mercaptoethyl)glycine-based monomer **11** was obtained in 32% overall yield.

Solid-Phase Synthesis of DNA-3′-PNA Chimeras **15**–**18**. Mmt-protected monomers **12** based on *N*-(2-aminoethyl)glycine and a universal 6-aminohexanol-derived CPG solid support **14** [10] were used for the synthesis of the PNA part of the DNA-3′-PNA (*Scheme 3*) [5][10][11]. After synthesis of the PNA part of the chimeras, the novel linker units **8** or **11**, respectively, were coupled at the junction to allow on-line synthesis of the DNA part of the chimeras by standard phosphoramidite chemistry [12], which, after cleavage from the support and deprotection, yielded the chimeras **15** and **17**, respectively. The DNA-3′-PNA chimera **16** was synthesized using the *N*-(2-hydroxyethyl)glycine-derived monomer **13** [13] as linker unit, while the phosphoramidate-linked chimera **18** was synthesized with the standard PNA monomer **12** and subsequent coupling of a protected nucleoside phosphoramidite to the terminal amino group of the PNA [9]. All DNA-3′-PNA chimeras were synthesized at 2-μmol scale. Deprotection of the Mmt or Dmt group was carried out with 3% trichloroacetic acid (TCA) in CH_2Cl_2 including one intermediate washing step with MeCN and subsequent neutralization of the support with $EtN(i\text{-}Pr)_2$ (DIPEA; or *Huenig*'s base). For efficient coupling of the monomers, a pre-activation step with either *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *O*-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), or (7-aza-1*H*-benztriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) in the presence of DIPEA was performed, whereby the required pre-activation time strongly depended

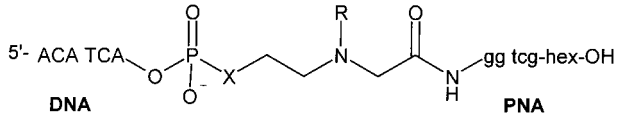
Scheme 3


1. 3% TCA
2. tetrazole
DNA synthesis
1. 3% TCA
2. HBTU, DIPEA
PNA synthesis
HBTU, DIPEA
X R2
11 S Dmt
12 NH Mmt
13 O Dmt
14

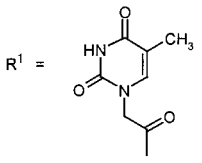
on the type of coupling reagent. While HATU and PyAOP gave sufficient coupling efficiencies (90–96%) using short pre-activation times (10–60 s), HBTU required longer pre-activation (15 min) to obtain similar results. Unreacted amino functions were capped with the standard DNA-capping mixture consisting of Ac_2O/*N*-methylimidazole in THF to prevent the growth of failure sequences due to incomplete coupling reactions.

The orthogonally protected monomeric PNA building blocks with base-labile protecting groups at the heterocyclic bases and an acid-labile Mmt group for temporary protection of the terminal amino function, or a Dmt group for the OH and SH functions, respectively, are fully compatible with standard phosphoramidite DNA synthesis. Consequently, the protected chimeras could be cleaved from the solid support (2 h at 50°) and simultaneously deprotected by treatment with concentrated aqueous NH_3 (16 h at 50°). The crude products were purified by denaturing preparative polyacrylamide gel electrophoresis (PAGE) and desalted *via C-18* columns. All DNA-3′-PNA chimeras were characterized by ion-exchange HPLC using a *Gen Pack Fax* column (*Millipore-Waters*) and by negative-ion electrospray mass spectroscopy (see *Exper. Part*).

Binding Affinity of the DNA-3′-PNA Chimeras. To study the influence of the type of linking unit at the DNA/PNA junction on the binding affinity of the chimeras, the UV melting curves of the corresponding duplexes with DNA and RNA were measured at 260 nm under physiological salt conditions (140 mM KCl, 10 mM NaH_2PO_4, 0.1 mM Na-EDTA, pH 7.4) and the T_m values were calculated. All three DNA-3′-PNA chimeras **16**–**18**, which have a nucleobase at the DNA/PNA junction, form more stable duplexes with complementary DNA **20** or RNA **21** than the natural oligonucleotide **19** (*Table 1*). The duplexes with complementary DNA were stabilized by +1.7 to +4.3 K, whereas the stabilization for complementary RNA was even larger (ΔT_m +4.7 to +6 K). The influence of X in the linking unit on duplex stabilization was in the order O > S ≈ NH, rendering the phosphodiester bridge the most favored linkage at the DNA/PNA junction. If the chimera was linked with just *N*-(2-hydroxyethyl)glycine without a nucleobase at the junction, such as in oligomer **15**, then the duplex with complementary DNA was destabilized by −7.1 K relative to the natural duplex, and by as much as −11.4 K relative to the DNA-PNA chimera **16** with a thymine base at the junction. This result implies that the nucleobase at the PNA unit of the junction actually forms a base pair with the complementary base of the second strand.



15 - 18



	X	R
15	O	H
16	O	R^1
17	S	R^1
18	NH	R^1

Table 1. T_m[a]) *Values of Duplexes of DNA-3′-PNA Chimeras* **15**–**18** *with Complementary DNA or RNA*

			T_m [°C]	
	X	R	DNA[b])	RNA[c])
15	O	H	42.8	43.3
16	O	R[1]	54.2	54.0
17	S	R[1]	52.1	52.7
18	NH	R[1]	51.6	53.0
19[d])	–	–	49.9	48.0

[a]) 140 mM KCl, 10 mM NaH_2PO_4, 0.1 mM Na-EDTA, pH 7.4
[b]) Complementary DNA: 3′-T G T A G T A C C A G C-5′ (**20**)
[c]) Complementary RNA: 3′-U G U A G U A C C A G C-5′ (**21**)
[d]) Unmodified oligonucleotide: 5′-A C A T C A T G G T C G-3′ (**19**)

To further analyze the contribution of the nucleobase-containing linker unit, we also measured the T_m values of the different chimeras **15**–**18** (*Table 2*) against complementary DNA having one base mismatch opposite to the base at the linking unit (mm-DNA; **22**) and against an oligonucleotide lacking the nucleobase at the junction (ab-DNA; **23**). Interestingly, introduction of a base mismatch destabilized the duplex to a similar extent (ΔT_m −7.7 to −11.5 K) as the removal of the nucleobase of the linking PNA unit (ΔT_m −11.4 K). Incorporation of an abasic unit in the complementary strand (ab-DNA; **23**) directly opposite to the linker unit confirmed the contribution of the nucleobase to binding: the T_m values of the duplexes with a base mismatch were between 40.6 to 45.4°, while the T_m values of the duplexes with an abasic unit were between 45.1 to 47.3°. Remarkably, the DNA · DNA duplex **23** · **24** with two abasic sites opposite to each other was significantly less stable (ΔT_m −14.5 K) than the DNA · DNA-PNA duplex **15** · **23**, in which the abasic site of the linker unit is opposite to the abasic site in the complementary DNA.

In conclusion, all investigated DNA-3′-PNA chimeras with a nucleobase at the junction formed more stable duplexes with complementary DNA and RNA than the corresponding unmodified DNA. The destabilizing effect of base mismatches or abasic

Table 2. *Influence of Base-Mismatches or Abasic Sites on the* T_m *Values*

			T_m [°C]		
	X	R	DNA[a])	mm-DNA[b])	ab-DNA[c])
15	O	H	42.8	45.6	46.0
16	O	R[1]	54.2	45.4	47.3
17	S	R[1]	52.1	40.6	45.5
18	NH	R[1]	51.6	43.9	45.1
19[d])	–	–	49.9	–	31.5
24[e])	–	–	34.0	–	31.5

[a]) Complementary DNA: 3′-T G T A G T A C C A G C-5′ (**20**)
[b]) Complementary mismatch-DNA: 3′-T G T A G T T C C A G C-5′ (**22**)
[c]) Complementary abasic-DNA: 3′-T G T A G T D C C A G C-5′ (**23**)
[d]) Unmodified sense oligonucleotide: 5′-A C A T C A T G G T C G-3′ (**19**)
[e]) Abasic sense oligonucleotide: 5′-A C A T C A D G G T C G-3′ (**24**)
D: abasic linker derived from phosphoric acid mono-[2-(hydroxymethyl)tetrahydrofuran-3-yl] ester

sites opposite to the nucleobase at the DNA/PNA junction suggests that this base very likely contributes to duplex stabilization through a *Watson-Crick* base pair. Furthermore, the phosphoramidite linkage (X = NH) may be an attractive alternative to the phosphodiester linkage (X = O) in DNA-3′-PNA chimeras, especially when hybridized to complementary RNA, since no additional building block for its incorporation is required.

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Experimental Part

1. *General*. The peptide coupling reagents HATU/1-hydroxy-7-aza-1*H*-benzotriazole (HOAt), and PyAOP were purchased from *Perseptives Biosystems*, HBTU, (9*H*-fluoren-9-yl)methyl *N*-succinimidyl carbonate (Fmoc-ONSu) and aminopropyl CPG from *Fluka* (Neu-Ulm, Germany). Standard nucleoside phosphoramidites and the reagent for introduction of the abasic linker (*dSpacer phosphoramidite*®) were purchased from *Eurogentec* (Seraing, Belgium). TLC was carried out on *Merck DC Kieselgel 60 F-254* glass plates. HPLC Analysis of PNAs: on a *Beckman System Gold* HPLC system, with a NaCl gradient (buffer *A:* 10 mM NaH_2PO_4, 100 mM NaCl in MeCN/H_2O 1:4 (*v*/*v*), pH 6.8; buffer *B:* 10 mM NaH_2PO_4, 1.5M NaCl in MeCN/H_2O 1:4 (*v*/*v*); 0 to 30% *B* in 30 min). T_m Values [°C ± 0.3] were measured under approximately physiological salt conditions (140 mM KCl, 10 mM NaH_2PO_4, 0.1 mM Na-EDTA, pH 7.4) in the cooling phase from 90° to 10° with a temp. ramp of 0.3°/min^{-1} at 260 nm on a *Varian Carey 1 Bio* UV/VIS Spectrometer at 1 μM oligomer concentration. ^{1}H-NMR Spectra: at 270 MHz in the solvents indicated; chemical shifts (δ) in ppm downfield relative to the internal standard. MS: either fast atom bombardement (FAB), electrospray (ES), or direct chemical ionization (DCI). NBA: 3-Nitrobenzyl alcohol.

2. *Monomeric Building Units.* N-*(2-Hydroxyethyl)glycine* (**6**). Glyoxylic acid monohydrate (46 g, 500 mmol) was dissolved in H_2O (1 l), and 2-aminoethanol (30.2 ml, 500 mmol) was added under stirring and cooling. The mixture was treated with 10% Pd/C catalyst (10 g) and hydrogenated (10 bar) in the autoclave at r.t. Then, the catalyst was filtered off, and the filtrate was concentrated *in vacuo*. The residue was co-evaporated twice with a small amount of toluene. The crude product was triturated using 250 ml of hot MeOH, filtered off, washed with MeOH, and dried: 49.56 g (83%). M.p. 178–180°(dec.). TLC (silica gel; BuOH/AcOH/H_2O/AcOEt 1:1:1:1 (*v*/*v*/*v*/*v*)): R_f 0.36. ^{1}H-NMR (D_2O): 3.77 (2 H, (*m*, HOCH_2); 3.68 (*s*, CH_2COOH); 3.22 (*m*, CH_2NH). DCI-MS: 120 ([*M* + H]$^+$).

N-*[(9*H*-Fluoren-9-yl)methoxycarbonyl]*-N-*(2-hydroxyethyl)glycine* (**7**). $NaHCO_3$ (10.08 g, 120 mmol) was dissolved in H_2O (150 ml) and **6** (7.15 g, 60 mmol) was added under stirring. After a clear soln. was obtained, Fmoc-ONSu (20.22 g, 60 mmol) in dioxane (300 ml) was added dropwise. After the final addition, stirring was continued for 3 h at r.t. The soln. was filtered, and the filtrate was concentrated *in vacuo*. The residue was solved in H_2O (100 ml), and the pH was adjusted to 2 with an $KHSO_4$ soln. Then, the mixture was extracted with AcOEt (3 × 150 ml), the combined org. layers were washed with H_2O (4 × 50 ml), dried (Na_2SO_4), filtered, and evaporated *in vacuo*. Precipitation was achieved with (i-Pr)$_2$O. The precipitate was filtered off, recrystallized from AcOEt/(i-Pr)$_2$O, filtered, and dried: 15.8 g (77%) of **7**. M.p. 114–116° (dec.). TLC (silica gel; BuOH/AcOH/H_2O 3:1:1 (*v*/*v*/*v*)): R_f 0.62. ^{1}H-NMR ((D$_6$)DMSO): 12.71 (br. *s*, 1 H); 7.24–7.95 (*m*, 8 H); 4.63 (br. *s*, 1 H); 4.19–4.39 (*m*, 3 H); 3.92–4.11 (*m*, 2 H); 3.11–3.56 (*m*, 4 H). ES-MS (pos. mode): 342 ([*M* + H]$^+$).

N-*[(9*H*-Fluoren-9-yl)methoxycarbonyl]-N-[2-[(4-methoxyphenyl)diphenylmethoxy]ethyl]glycine* (**8**). Compound **7** (14.74 g, 43 mmol) was dissolved in pyridine (160 ml), (4-methoxyphenyl)diphenylmethyl chloride (13.31 g, 43 mmol) was added, and the mixture was stirred for 2 h at r.t. After evaporation *in vacuo*, the residue was taken up in AcOEt (300 ml) and extracted with sat. aq. $NaHCO_3$ (3 × 30 ml) and H_2O (3 × 30 ml). The org. layer was dried (Na_2SO_4), filtered, evaporated, and precipitated with (i-Pr)$_2$O. The product was filtered and dried. 19.95 g (70%) of **8**. TLC (silica gel; AcOEt/MeOH/Et_3N 60:40:1 (*v*/*v*/*v*)): R_f 0.45. ^{1}H-NMR ((D$_6$)DMSO): 6.80–7.95 (*m*, 22 H); 4.09–4.31 (*m*, 3 H); 3.80, 3.91 (2*s*, 2 H); 3.76 (*s*, 3 H); 2.92–3.56 (*m*, 4 H). FAB-MS (MeOH/NBA): 658.3 ([*M* + 2Na]$^+$).

N-*(2-[[Bis(4-methoxyphenyl)phenylmethyl]thio]ethyl)*-N-*[(thymin-1-yl)acetyl]glycine* (**11**). To a soln. of Dmt-Cl (10.17 g, 30 mmol) in AcOH (100 ml), 2-mercaptoethylamine hydrochloride (4.43 g, 39 mmol)

dissolved in H_2O (70 ml) was added. The soln. was stirred for 2 h at r.t. and then evaporated *in vacuo* to a volume of 50 ml. After addition of H_2O (200 ml), the pH was adjusted to 10 using 2N NaOH. The mixture was extracted with AcOEt (2 × 100 ml), and the combined org. phases were washed with sat. NaCl soln., dried, filtered, and concentrated *in vacuo* (14.75 g). Part of the resulting crude product (12.56 g) was dissolved in dry DMF (100 ml), treated with Et_3N (4.6 ml, 33 mmol) and ethyl bromoacetate (3.66 ml, 33 mmol) and stirred for 3 h. The soln. was concentrated *in vacuo*, and the residue was taken up in AcOEt (150 ml), washed with H_2O (4 × 40 ml). Then, the org. layer was dried (Na_2SO_4), filtered, and evaporated *in vacuo* (12.92 g). Part of the resulting crude product (**10**; 5.95 g) was dissolved in dry DMF (50 ml) and (thymin-2-yl)acetic acid (2.36 g, 12.8 mmol), Et_3N (4.45 ml, 32 mmol), and TOTU (4.2 g, 12.8 mmol) were added to the soln. The mixture was stirred for 2 h at r.t., then evaporated *in vacuo*. The residue was taken up in AcOEt (150 ml) and washed with sat. $NaHCO_3$ soln. (3 × 10 ml) and H_2O (2 × 10 ml). The org. layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The resulting residue was dissolved in a mixture of dioxane (80 ml) and H_2O (40 ml), and the ester was hydrolyzed by adding 2N NaOH soln. in portions (13 ml). The soln. was concentrated *in vacuo* to a volume of 50 ml and extracted with AcOEt (4 × 50 ml). The pH of the aq. layer was adjusted to 5 with 2N HCl, and the soln. was then extracted with AcOEt (4 × 50 ml). The combined org. layers were washed twice with H_2O (30 ml), dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The crude product was purified by silica-gel chromatography using a step gradient of 0–5% MeOH in CH_2Cl_2 (1% Et_3N in all eluents). The fractions containing the product were pooled and dried *in vacuo:* 2.38 g (32% from Dmt-Cl) of **11**. TLC (silica gel; CH_2Cl_2/MeOH/Et_3N 100 : 10 : 1 (*v*/*v*/*v*)): R_f 0.38. FAB-MS (NBA + LiCl): 610.2 ($[M+Li]^+$), 616.2 ($[M+2Li-H]^+$). ^{1}H-NMR ((D_6)DMSO): 11.4 (*s*, H–C(3) of thymine); 6.91–7.38 (*m*, 14 H, Dmt, H–C(6) of thymine); 4.16, 4.39 (2*s*, $NCOCH_2$); 3.69 (2*s*, 2 MeO); 3.56, 3.61 (2*s*, NCH_2COOH); 2.98 (*m*, CH_2N); 2.31 (*m*, CH_2S); 1.76 (*s*, Me of thymine).

2. *Solid-Phase Synthesis of the DNA-3′-PNA Chimeras.* The Mmt/acyl-protected monomer **12** and Dmt/acyl-protected monomer **13** were prepared as described in [13]. The Mmt-protected 6-aminohexan-1-ol/succinylamidopropyl CPG [10] with a loading 36 μmol/g was used as solid-support. Synthesis was performed on a modified *Eppendorf Biotronik Ecosyn D-300* DNA synthesizer or on an *ABI 394* DNA synthesizer at 2-μmol scale. The DNA part was synthesized according to standard procedures [12]. The following synthesis conditions were used for the synthesis of the PNA part including the *N*-(2-mercaptoethyl)glycine, *N*-(2-hydroxyethyl)-glycine, or *N*-(2-aminoethyl)glycine derived linkers: *1*) *washing step* with MeCN; *2*) *deprotection* of the Mmt group: 3% TCA in CH_2Cl_2; 110 s total treatment time interrupted by one wash with MeCN for 20 s; *3) washing step* with DMF/MeCN 1 : 1 (*v*/*v*); *4*) *syringe wash*; *5*) *neutralization:* washing with DMF/MeCN 1 : 1 (*v*/*v*) and $EtN(i\text{-}Pr)_2$; *6*) *coupling of monomers:* Monomers (0.2 to 0.3M solutions in DMF); $etN(i\text{-}Pr)_2$ (0.2 to 0.3M in DMF); coupling reagent (0.2–0.3M in DMF); reagents were pre-mixed, pre-activated and delivered onto the solid support. Due to the use of different coupling reagents, we had to adjust the pre-activation and reaction time; *7*) *capping*. Reagent *A:* 10% Ac_2O/10% lutidine in THF; reagent *B:* 16% *N*-methyl-1*H*-imidazole in THF (the DNA capping reagents *A* and *B* were mixed just before use).

After synthesis was complete, the chimeras were cleaved from the support (2.5 h at 50°) and deprotected (6 h at 50°) with concentrated aq. NH_3 soln. The crude product was analyzed by HPLC using a *Gen Pack Fax* column (*Millipore-Waters*), eluting with a NaCl gradient (buffer *A:* 10 mM NaH_2PO_4, 100 mM NaCl in MeCN/H_2O 1 : 4 (*v*/*v*) pH 6.8; buffer *B:* 10 mM NaH_2PO_4, 1.5M NaCl in MeCN/H_2O 1 : 4 (*v*/*v*); 0–30% *B* in 30 min). Purification was achieved by prep. PAGE (15% polyacrylamide) and desalted *via* a *C-18* column. The purified PNA/DNA chimeras were further analyzed by negative-ion ES-MS.

3. *Synthesis of 5′-ACA TCA oeg*(X = O, R = H)*gg tcg-hex-OH* (**15**). As described in *Exper. 2*, with HATU/HOAt (0.3M) as coupling reagent with a pre-activation time of 1 min, and a total coupling time of 45 min. Yield of crude product was 101 OD. Purification of 42 OD of the crude product resulted in 3.2 OD purified product. Characterization by negative-ion ES-MS: 3473.75 ± 0.13 (*M*; calc. for $C_{124}H_{162}N_{55}O_{54}P_6$: 3473.86).

4. *Synthesis of 5′-ACA TCA oeg-t*(X = O, R = R^1)*gg tcg-hex-OH* (**16**). As described in *Exper. 2*, with HBTU (0.2M) as coupling reagent with permanent pre-activation (DNA synthesizer *ABI 394*), and a total coupling time of 15 min. Yield of crude product was 26 OD. Purification of the crude product (26 OD) resulted in 13.6 OD purified product. Characterization by negative-ion ES-MS: 3596.65 ± 0.77 (*M*; calc. for $C_{129}H_{167}N_{57}O_{56}P_6$: 3597.94).

5. *Synthesis of 5′-ACA TCA seg-t*(X = S, R = R^1)*gg tcg-hex-OH* (**17**). As described in *Exper. 2*, with HBTU (0.25M) as coupling reagent with a pre-activation time of 15 min, and a total coupling time of 15 min. Yield of crude product was 60 OD. Purification of 44 OD of the crude product resulted in 9.5 OD purified product. Characterization by negative-ion ES-MS: 3613.51 ± 0.13 (*M*; calc. for $C_{129}H_{167}N_{57}O_{55}P_6S$: 3614.00).

6. *Synthesis of 5′ACA TCA aeg-t*(X = NH, R = R[1])*gg tcg-hex-OH* (**18**). As described in *Exper. 2*, with PyAOP (0.3M) as coupling reagent with a pre-activation time of 10 s, and a total coupling time of 15 min. Yield of crude product was 52 OD. Purification of 51 OD of the crude product resulted in 1.9 OD of purified product. Characterization by negative-ion ES-MS: 3596.49 ± 0.09 (*M*; calc. for $C_{129}H_{168}N_{58}O_{55}P_6$: 3596.98).

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