(2′-*O*-Methyl-RNA)-3′-PNA Chimeras: A New Class of Mixed Backbone Oligonucleotide Analogues with High Binding Affinity to RNA

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Dedicated to Prof. Dr. Dr. *h.c. Wolfgang Pfleiderer* on the occasion of his 75th birthday

The automated on-line synthesis of DNA-3′-PNA chimeras **1**–**4** and (2′-*O*-methyl-RNA)-3′-PNA chimeras **5**–**8** is described, in which the 3′-terminal part of the oligonucleotide is linked to the N-terminal part of the PNA *via* *N*-(ω-hydroxyalkyl)-*N*-[(thymin-1-yl)acetyl]glycine units (alkyl = Et, Ph, Bu, and pentyl). By means of UV thermal denaturation, the binding affinities of all chimeras were directly compared by determining their T_m values in the duplex with complementary DNA and RNA. All investigated DNA-3′-PNA chimeras and (2′-*O*-methyl-RNA)-3′-PNA chimeras form more-stable duplexes with complementary DNA and RNA than the corresponding unmodified DNA. Interestingly, a *N*-(3-hydroxypropyl)glycine linker resulted in the highest binding affinity for DNA-3′-PNA chimeras, whereas the (2′-*O*-methyl-RNA)-3′-PNA chimeras showed optimal binding with the homologous *N*-(4-hydroxybutyl)glycine linker. The duplexes of (2′-*O*-methyl-RNA)-3′-PNA chimeras and RNA were significantly more stable than those containing the corresponding DNA-3′-PNA chimeras. Surprisingly, we found that the charged (2′-*O*-methyl-RNA)-3′-PNA chimera with a *N*-(4-hydroxybutyl)glycine-based unit at the junction to the PNA part shows the same binding affinity to RNA as uncharged PNA. Potential applications of (2′-*O*-methyl-RNA)-3′-PNA chimeras include their use as antisense agents acting by a RNase-independent mechanism of action, a prerequisite for antisense-oligonucleotide-mediated correction of aberrant splicing of pre-mRNA.

Introduction. – The strong and sequence-specific binding of peptide or polyamide nucleic acids (PNAs) to complementary DNA and RNA has rendered them attractive candidates for the development of novel therapeutics and diagnostics [1–3]. Recently, we have shown that some limitations of PNA, such as low cellular uptake, the inability to activate RNase H, and poor solubility, can be overcome by the use of DNA-PNA chimeras [4]. Although these DNA-PNA chimeras form more-stable complexes with complementary DNA and RNA than the corresponding DNA, their binding affinities are lower than those of the pure PNAs. In the previously described DNA-3′-PNA chimeras, binding affinity was highest when the 3′-part of the oligodeoxynucleotide was linked to a terminal *N*-(2-hydroethyl)glycine unit of the PNA *via* a phosphodiester unit [5].

Since the type of junction in the DNA-PNA chimeras appeared to significantly influence binding affinity [5][6], we aimed to further optimize the linking moiety at the junction of DNA and PNA by systematically varying the length of the alkyl chain of the *N*-(ω-hydroxyalkyl)glycine unit (alkyl = Et, Pr, Bu, and pentyl). As a second variable, we altered the sugar residue within the charged oligonucleotide part of the chimera so

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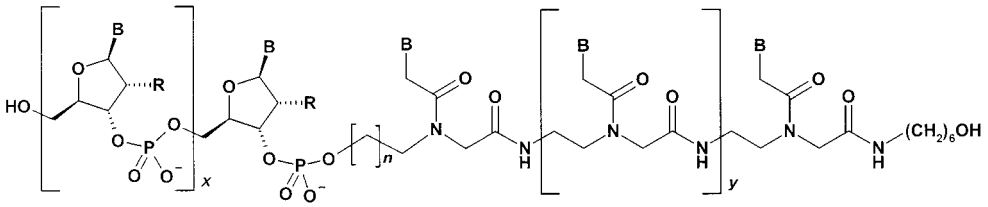
as to achieve a higher pre-organization of the single-stranded chimera, resulting in an entropic gain on duplex formation. Certain RNA analogues, such as 2′-*O*-methyl-RNA, in which the 2′-*O*-Me group is in the 2′-*exo* conformation, form a A-type helix with complementary RNA leading to enhanced binding affinity as compared to the 2′-deoxyribonucleotide analogue [7]. Although RNase H-incompetent oligonucleotide analogs, such as PNA, morpholino oligomers, and 2′-*O*-alkyl oligoribonucleotides, are usually less-efficient antisense inhibitors of translation than RNase H-competent antisense oligomers when targeted downstream of the AUG translational start signal, the RNase H-independent antisense types can inhibit translation very efficiently through a steric blocking mechanism when targeted against sequences in the mRNA region between the 5′-cap and the AUG start site [8][9]. Therefore, (2′-*O*-methyl-RNA)-3′-PNA chimeras are of interest as potential antisense agents acting by a RNase H-independent mechanism of action, a prerequisite for antisense-oligonucleotide-mediated correction of aberrant splicing of pre-mRNA. Here, we describe the synthesis and binding affinity of both DNA-3′-PNA and (2′-*O*-methyl-RNA)-3′-PNA chimeras, **1**–**8**, with linking units of varying length. To guide the optimal future design of these chimeric molecules, we directly compare the binding affinity of DNA-3′-PNA and (2′-*O*-methyl-RNA)-3′-PNA chimeras by measurement of the thermal denaturation curves of the various chimeras.

Results and Discussion. – *Synthesis of the Monomeric Linker Units* **12a**–**h** (*Scheme*). The *N*-(ω-hydroxyalkyl)glycine linker units of varying length ($n = 1$, 2, 3, and 4), which are required to form the DNA-PNA junction of the chimeras, were synthesized by a method previously described for *N*-(2-hydroxyethyl)glycine derivative ($n = 1$) [5]. The *N*-(ω-hydroxyalkyl)glycine intermediates are prepared in *ca.* 80% yield by reductive amination of the corresponding ω-aminoalkyl alcohol with glyoxylic acid in H_2O with H_2 as reducing agent and Pd/C as catalyst [10], which were then used without further purification in the condensation reaction with thymine-3-acetic acid with *O*-{[(2-cyanoethoxycarbonyl)methylidene]amino}-1,1,3,3-tetramethyluronium tetrafluoroborate (TOTU) as activating agent. Finally, the dimethoxytrityl-protected linker units **12e**–**h** were obtained by reaction of the OH group of the hydroxyalkyl intermediates with bis(4-methoxyphenyl)phenylmethyl (dimethoxytrityl, Dmt) chloride in CH_2Cl_2. After purification of the crude product by silica-gel chromatography with 1% Et_3N in CH_2Cl_2/MeOH/AcOEt, the *N*-(ω-hydroxyalkyl)glycine-based monomers **12e**–**h** could be isolated in *ca.* 30–50% overall yield.

*Solid-Phase Synthesis of DNA-3′-PNA and (2′-*O*-Methyl-RNA)-3′-PNA Chimeras* **1**–**8**. *N*-Mmt-protected (Mmt = monomethoxytrityl) PNA monomers and CPG solid support were used for the synthesis of DNA-3′-PNA and (2′-*O*-methyl-RNA)-3′-PNA chimeras on a modified *Eppendorf Biotronik Ecosyn D-300* DNA synthesizer [11]. The chimeras were synthesized on a 2 μmol scale, starting with the synthesis of the PNA part. Deprotection of the temporary Mmt group of the growing chain was carried out with 3% Cl_3CCOOH in CH_2Cl_2, including one intermediate washing step with MeCN. Then, the solid support was neutralized with $EtN(i\text{-}Pr)_2$ (0.2–0.3M in DMF). For the coupling reaction of the monomer (0.2–0.3M solution in DMF), a pre-activation step with HATU (*O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)/HOAt (1-hydroxy-7-azo-1*H*-benzotriazole), HBTU (*O*-(1*H*-benzotriazol-1-

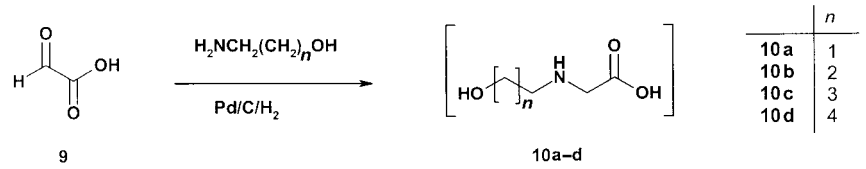


	n	R
1	1	H
2	2	H
3	3	H
4	4	H
5	1	MeO
6	2	MeO
7	3	MeO
8	4	MeO

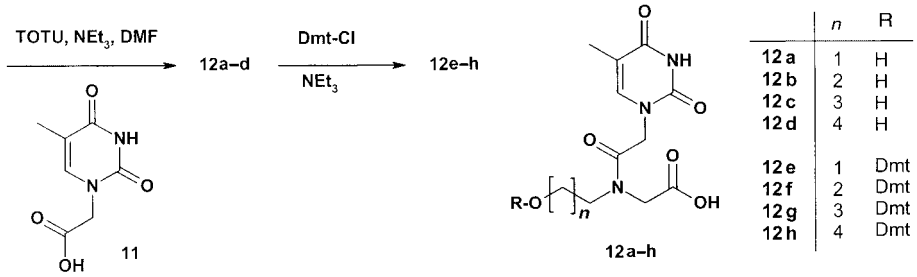
R = H, MeO $x = 5$, $y = 4$

B = adenine, guanine, cytosine, or thymine

Scheme



	n
10a	1
10b	2
10c	3
10d	4



	n	R
12a	1	H
12b	2	H
12c	3	H
12d	4	H
12e	1	Dmt
12f	2	Dmt
12g	3	Dmt
12h	4	Dmt

yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or PyAOP (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (0.2 – 0.3M in DMF) in the presence of $EtN(i\text{-}Pr)_2$ (0.2 – 0.3M in DMF) is inserted. The optimal pre-activation time depends on the type of coupling reagent. For example, HATU and PyAOP afford only short pre-activation times (10 s), whereas for HBTU a longer activation (15 min) is advantageous. After pre-activation, the mixture containing the activated PNA monomer is delivered to the reaction vessel for coupling (reaction times between 15 and 45 min). Unreacted amino functions are capped with the standard DNA capping mixture consisting of Ac_2O/*N*-methylimidazole in THF. At the junction between PNA and the charged nucleic acid part, the Dmt-protected *N*-(ω-hydroxyalkyl)glycine-based

linking moieties are coupled in a similar manner to the (2-aminoethyl)glycine-based PNA monomers. After removal of the Dmt-group with 3% Cl_3CCOOH in CH_2Cl_2, standard 2′-deoxynucleotide phosphoramidites or 2′-*O*-methylribonucleotide phosphoramidites are coupled to the OH group of the growing chain by means of tetrazole. After synthesis is complete, the chimera is cleaved from the solid support (2 h at 50°) and deprotected with concentrated aqueous NH_3 (16 h at 50°). The crude product is purified by denaturing preparative PAGE (15% PPA) [12], desalted *via* a *C-18* cartridge and characterized by ion-exchange HPLC on a *Gen Pack Fax* column (*Millipore-Waters*) and by negative-ion electrospray mass spectrometry.

*Binding Affinity of the DNA-3′-PNA Chimeras and (2′-*O*-Methyl-RNA)-3′-PNA Chimeras.* Thermal denaturation was employed to study the influence of the type of sugar in the nucleic acid part and the type of linking unit at the DNA-3′-PNA or (2′-*O*-methyl-RNA)-3′-PNA junction, respectively, on the binding affinity of the chimeras. For this, the UV melting curves of the corresponding duplexes with DNA and RNA were measured at 260 nm under physiologic 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 four different DNA-3′-PNA chimeras **1**–**4** form more-stable duplexes with complementary DNA **17** or RNA **18** than the natural oligonucleotide **16** (*Table 1*). We found that the linker unit at the DNA-PNA junction consisting of a *N*-(3-hydroxypropyl)glycine unit ($n=2$) results in highest binding affinity of the four spacers of different length. The duplexes of the DNA-3′-PNA chimeras were stabilized by up to +5.6 K with DNA **17** as complementary strand and by up to +6.8 K with complementary RNA **18**. The DNA-3′-PNA chimeras **1**–**4** retain their base specificity regardless of the type of linking moiety as shown by the lowered T_m values of their duplexes with a complementary strand **19** having one base-pair mismatch. Interestingly, the duplexes of (2′-*O*-methyl-RNA)-3′-PNA chimeras **5**–**8** with complementary DNA were stabilized by only 0.2–2.7 K relative to the natural duplex, whereas, on hybridization to complementary RNA, a significant enhancement of binding affinity by an increase of +14 to +16.6 K relative to the natural DNA-RNA duplex is observed (*Table 2*). As found for the DNA-3′-PNA chimeras **1**–**4**, there is also an optimal linker length for the (2′-*O*-methyl-RNA)-3′-PNA chimeras **5**–**8**, which is, however, based on the homol-

Table 1. T_m[a]) *Values of Duplexes of DNA-3′-PNA Chimeras* **1**–**4** *with Complementary DNA, RNA, and Mismatched DNA*

	n	R	T_m [°] DNA[c])	T_m [°] RNA[d])	ΔT_m [K] mmDNA[e])
1	1	H	54.2 ± 0.3	54.0 ± 0.4	8.5
2	2	H	55.5 ± 0.3	54.8 ± 0.3	9.1
3	3	H	54.0 ± 0.4	53.8 ± 0.5	9.1
4	4	H	53.8 ± 0.1	53.8 ± 0.3	8.7
16[b])	–	–	49.9 ± 0.4	48.0 ± 0.4	7.3

[a]) 140 mM KCl, 10 mM NaH_2PO_4, 0.1 mM Na-EDTA, pH 7.4.
[b]) Unmodified oligonucleotide: 5′-ACA TCA TGG TCG-3′ (**16**)
[c]) Complementary DNA: 3′-TGT AGT ACC AGC-5′ (**17**)
[d]) Complementary RNA: 3′-UGU AGU ACC AGC-5′ (**18**)
[e]) Complementary mismatch strand (mmDNA): 3′-TGT AGT TCC AGC-5′ (**19**)

Table 2. T_m[a]) *Values of Duplexes of (2′-*O*-Methyl-RNA)-3′-PNA Chimeras* **5**–**8** *with Complementary DNA and RNA*

	n	R	T_m [°] DNA[c])	T_m [°] RNA[d])
5	1	MeO	50.4 ± 0.3	61.1 ± 0.4
6	2	MeO	50.1 ± 0.4	60.5 ± 0.1
7	3	MeO	52.6 ± 0.2	63.1 ± 0.4
8	4	MeO	50.6 ± 0.1	61.1 ± 0.5
20[b])	–	–	58.5 ± 0.4	62.3 ± 0.5

[a]) 140 mM KCl, 10 mM NaH_2PO_4, 0.1 mM Na-EDTA, pH 7.4.
[b]) Uniform PNA: 5′-a c a t c a t g g t c g-3′ (**20**)
[c]) Complementary DNA: 3′-TGT AGT ACC AGC-5′ (**17**)
[d]) Complementary RNA: 3′-UGU AGU ACC AGC-5′ (**18**)

ogous *N*-(4-hydroxybutyl)glycine unit ($n = 3$). Surprisingly, the binding affinity of the multiply charged (2′-*O*-methyl-RNA)-3′-PNA chimera **7** to complementary RNA is equal to that of the corresponding uncharged PNA **20**.

In conclusion, all investigated DNA-3′-PNA chimeras and (2′-*O*-methyl-RNA)-3′-PNA chimeras form more stable duplexes with complementary DNA and RNA than the corresponding unmodified DNA. While the DNA-PNA chimeras show almost the same binding affinity both with DNA and RNA as the complementary target, the (2′-*O*-methyl-RNA)-3′-PNA chimeras have a clear preference for RNA as the complementary target sequence. In this respect, it is interesting to note that charge repulsion in the duplex of (2′-*O*-methyl-RNA)-3′-PNA chimeras does not dominate binding affinity to complementary RNA, since the charged (2′-*O*-methyl-RNA)-3′-PNA chimera binds with affinity equal to that of the completely uncharged PNA. The different structural characteristics of the duplexes – PNA-containing duplexes form a wider helix than their natural counterparts [3] – may become apparent in the different spacer length required for achieving optimal binding affinity. Thus, a *N*-(3-hydroxypropyl)glycine linker was optimal for DNA-3′-PNA chimeras, whereas the (2′-*O*-methyl-RNA)-3′-PNA chimeras showed optimal binding with the homologous *N*-(4-hydroxybutyl)glycine linker. Taken together, this is the first report of a charged PNA analog having the same binding affinity to RNA as uncharged PNA itself. The (2′-*O*-methyl-RNA)-3′-PNA chimeras should be very useful for RNase H-independent antisense inhibition of gene expression. Antisense inhibitors that work by an RNase H-independent mechanism are attracting increasing interest for pharmaceutical target-validation experiments and for redirecting splicing events [8]. To allow inhibition of splicing of the pre-mRNA by antisense oligonucleotide analogues, derivatives that do not stimulate RNase H must be used in these experiments, such as morpholino-type analogs, PNA, or (2′-*O*-methyl-RNA)-3′-PNA chimeras.

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Experimental Part

1. *General*. The peptide-coupling reagents HATU/HOAt and PyAOP were purchased from *Perseptive Biosystems*, HBTU, (9*H*-fluoren-9-yl)methyl-*N*-succinimidyl carbonate (Fmoc-ONSu), dimethoxytrityl chloride, and aminopropyl CPG from *Fluka* (Neu-Ulm, Germany). Standard nucleoside phosphoramidites were obtained from *Eurogentec* (Seraing, Belgium). TLC Analysis was performed with *Merck* DC silica-gel *60 F-254* glass plates. HPLC Analysis of PNAs was carried out on a *Beckman System Gold* HPLC system, 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 to 30% *B* in 30 min). ^{1}H-NMR Spectra were recorded at 270 MHz in the solvents indicated. Chemical shifts (δ) are reported in ppm downfield relative to the internal standard. The T_m values ($^\circ \pm 0.3$) were measured under pseudo-physiologic 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 0.3°/min^{-1} at 260 nm on a *Varian Carey 1 Bio* UV/VIS spectrometer at 1-μM oligomer concentration. Mass spectra were recorded with either fast-atom bombardment (FAB), electrospray (ES), or direct chemical ionization (DCI).

2. *Monomeric Building Units.* The (ω-hydroxyalkyl)glycines **10b**–**d** were synthesized according the *N*-(2-hydroxyethyl)glycine procedure as described previously for preparation of **10a** [5] by reductive amination of the corresponding (ω-hydroxyalkyl)amines with glyoxylic acid, H_2 as reducing agent and Pd/C as catalyst.

General Procedure 1 (*GP 1*) *for the Coupling*. To a soln. of thymine-3-acetic acid (1 equiv.) in dry DMF, TOTU (1 equiv.) and Et_3N (1 equiv.) were added. This mixture was added, after stirring for 30 min, dropwise to a soln. composed of *N*-(ω-hydroxyalkyl)glycine (2 equiv.), H_2O, DMF, and Et_3N (2 equiv.), stirred further at r.t., and then evaporated *in vacuo*. The residue was dissolved in H_2O, adjusted to pH 1.5 with 1N HCl, and extracted with AcOEt. The aq. layer was adjusted to pH 5 with sat. $NaHCO_3$ soln. and concentrated *in vacuo*. The residue was then mixed with 250 ml of EtOH. The precipitated NaCl was filtered off, and the filtrate was concentrated *in vacuo*. The crude product was purified by chromatography on silica gel with CH_2Cl_2/MeOH/AcOEt with the addition of 1% of Et_3N.

N-*(3-Hydroxypropyl)*-N-*[(thymin-1-yl)acetyl]glycine* (**12b**) was synthesized according to the *GP 1* in a 20-mmol scale. The crude product was purified with CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) and the addition of 1% of Et_3N. The fractions containing the product were combined and dried *in vacuo:* 3.2 g (53%) of **12b**. TLC (silica gel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) + 1% Et_3N): R_f 0.15). ^{1}H-NMR (D_2O): 7.40 (*m*, H–C(6) of thymine), 4.81 + 4.59 (2*s*, $NCOCH_2$ (rotamers)); 4.02 + 3.95 (2*s*, NCH_2COOH (rotamers)); 3.41–3.65 (*m*, CH_2O, NCH_2)); 1.87 (*s*, Me of thymine); 1.41–1.89 (*m*, CH_2). ES$^+$-MS: 300.2 ($[M+H]^+$).

N-*(4-Hydroxybutyl)*-N-*[(thymin-1-yl)acetyl]glycine* (**12c**) was prepared in a 15-mmol scale according to *GP 1*. The crude product was purified with CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) and the addition of 1% of Et_3N. The fractions containing the product were combined and dried *in vacuo:* 3.7 g (79%) of **12c**. TLC (silica gel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) + 1% Et_3N): R_f 0.11. ^{1}H-NMR (D_2O) 7.39 (*m*, H–C(6) of thymine); 4.79, 4.58 (2*s*, $NCOCH_2$ (rotamers)); 4.03, 3.91 (2*s*, NCH_2COOH (rotamers)); 3.25–3.65 (*m*, CH_2O, NCH_2)); 1.89 (*s*, Me of thymine); 1.31–1.86 (*m*, $(CH_2)_2$). ES$^+$-MS: 314.2 ($[M+H]^+$).

N-*(5-Hydroxypentyl)*-N-*[(thymin-1-yl)acetyl]glycine* (**12d**) was synthesized according to *GP 1* in a 15-mmol scale. The crude product was purified with CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) and the addition of 1% of Et_3N. The fractions containing the product were combined and dried *in vacuo:* 3.34 g (68%) of **12d**. TLC (silica cel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) + 1% Et_3N): R_f 0.19. ^{1}H-NMR (D_2O) 7.41 (*m*, H–C(6) of thymine), 4.80, 4.59 (2*s*, $NCOCH_2$ (rotamers)); 4.00, 3.90 (2*s*, NCH_2COOH (rotamers)); 3.29–3.69 (*m*, 4 H, (O–CH_2; N–CH_2)); 1.89 (*s*, Me of thymine)); 1.29–1.69 (*m*, 6 H, $(CH_2)_3$). DCI-MS: 328.2 ($[M+H]^+$).

General Procedure 2 (*GP 2*) *for the Tritylation*. To a mixture of *N*-(ω-hydroxyalkyl)-*N*-[(thymin-1-yl)acetyl]glycine (1 equiv.) and Et_3N (4–5 equiv.) in DMF, a soln. of Dmt-Cl (2–4 equiv.) in CH_2Cl_2 was added dropwise at 0° and stirred further at r.t. The precipitated Et_3N was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and extracted with H_2O. The org. layer was dried (Na_2SO_4) and concentrated *in vacuo*. The crude products were purified on silica gel with CH_2Cl_2/MeOH/AcOEt 10:2:1 and the addition of 1% Et_3N.

N-*{3-[Bis(4-methoxyphenyl)phenylmethoxy]propyl}*-N-*[(thymin-1-yl)acetyl]glycine* (*Dmt-opg(t)-OH*; **12f**) was synthesized according to *GP 2* in a 10.7-mmol scale. The crude products were purified by chromatography on silica gel with CH_2Cl_2/MeOH/AcOEt 10:2:1 and the addition of 1% Et_3N: 3.46 g (54%) of **12f**. TLC (silica gel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v/v/v*) + 1% Et_3N): R_f 0.28. ^{1}H-NMR ((D_6)DMSO): 11.22 (*s*, H–N(3) of thymine); 6.86–7.40 (*m*, 15 H, Dmt, H–C(6) of thymine); 4.61, 4.40 (2*s*, $NCOCH_2$ (rotamers)); 3.62–3.81 (3*s*, NCH_2COOH (rotamers), 2 MeO); 2.91–3.11 (*m*, CH_2O, NCH_2); 1.76 (*s*, Me of thymine); 1.63–1.81 (*m*, CH_2). ES$^+$-MS: 602.4 ($[M+H]^+$).

N-*{4-[Bis(4-methoxyphenyl)phenylmethoxy]butyl}*-N-*[(thymin-1-yl)acetyl]glycine* (*Dmt-obg(t)-OH*, **12g**) was synthesized according to *GP 2* in a 11.4-mmol scale. The crude products were purified on silica gel with CH_2Cl_2/MeOH/AcOEt 15:1:1 and the addition of 1% Et_3N: 3.45 g (49%) of **12g**. TLC (silica gel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v*/*v*/*v*) + 1% Et_3N): R_f 0.29. ^{1}H-NMR ((D$_6$)DMSO): 11.23 (*m*, H–N(3) of thymine); 6.82–7.44 (*m*, 15 H, Dmt, H–C(6) of thymine); 4.52, 4.43 (2*s*, $NCOCH_2$ (rotamers)); 3.72–3.85 (3*s*, NCH_2COOH (rotamers), 2 MeO); 2.80–3.31 (2*m*, CH_2O, NCH_2)); 1.75 (*s*, Me of thymine); 1.41–1.65 (*m*, $(CH_2)_2$). ES$^+$-MS (LiCl): 622.3 ($[M+Li]^+$).

N-*{5-[Bis(4-methoxyphenyl)phenylmethoxy]pentyl}*-N-*[(thymin-1-yl)acetyl]glycine* (*Dmt-opeg(t)-OH*; **12h**) was synthesized according to *GP 2* in a 9.78-mmol scale. The mixture is stirred for further 2 h. The crude products were purified on silica gel with CH_2Cl_2/MeOH/AcOEt 15:1:1 and the addition of 1% Et_3N: 3.6 g (59%) of **12h**. TLC (silica gel; CH_2Cl_2/MeOH/AcOEt 10:2:1 (*v*/*v*/*v*) + 1% Et_3N): R_f 0.27. ^{1}H-NMR ((D$_6$)DMSO): 11.22 (*m*, H–N(3) of thymine); 6.83–7.39 (*m*, 15 H, Dmt, H–C(6) of thymine); 4.59, 4.41 (2*s*, $NCOCH_2$ (rotamers)); 3.73–3.85 (3*s*, NCH_2COOH (rotamers), 2 MeO); 2.89–3.35 (2*m*, CH_2ONCH_2)); 1.76 (*d*, Me of thymine), 1.22–1.60 (*m*, $(CH_2)_3$). ES$^+$MS(LiCl): 636.4 ($[M+Li]^+$).

3. *Solid-Phase Synthesis.* Mmt/Acyl-protected monomers were used for synthesis of the PNA part of the chimeras as described in [13][10]. As solid support, we employed the Mmt-protected 6-aminohexan-1-ol/succinylamidopropyl CPG with a loading of 36 μmol/g [13]. Synthesis was performed on a modified *Eppendorf Biotronik Ecosyn D-300* and *ABI 394* DNA synthesizer at a 2-μmol scale. The DNA part was synthesized according to the standard phosphoramidite chemistry [14]. The following synthesis conditions were used for the synthesis of the PNA part including the *N*-(*ω*-hydroxyalkyl)glycine-derived linker: 1) *washing step* with MeCN; 2) *deprotection step* of the Mmt group: 3% Cl_3COOH 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-Pr)$_2$; 6) *coupling step of monomers:* monomers (0.2 to 0.3M solns. in DMF); EtN(i-Pr)$_2$ (0.2 to 0.3M in DMF); coupling reagent (0.2 to 0.3M in DMF); reagents were premixed, pre-activated and delivered onto the solid support; 7) *capping step:* reagent *A:* 10% Ac_2O/10% lutidine in THF; reagent *B:* 16% *N*-methyl-1*H*-imidazole in THF.

After completion of the synthesis, the fully protected chimeras were cleaved from the support (2.5 h at 50°) and deprotected (6 h at 50°) with conc. aq. NH_3 soln. The resulting crude product was analyzed by HPLC chromatography with a *Gen Pack Fax* column (*Millipore-Waters*) and 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% in 30 min) as eluent. Finally, purification of the chimeras was achieved by prep. PAGE (15% polyacrylamide), and the products obtained were desalted *via* a *C-18* column. The purified DNA-3′-PNA and (2′-*O*-methyl-RNA)-3′-PNA chimeras were further characterized by ES-MS (neg. ion).

4. *Synthesis of 5′-ACA TCA t(oeg)gg tcg-hex-OH* (**1**; $n=1$, R = H). As described in *Exper. 3*, with HBTU (0.2M) as couping reagent, with a permanent pre-activation time (DNA synthesizer *ABI 394*), and a total coupling time of 15 min. Yield of the crude product 26 OD. Purification of 26 OD of the crude product resulted in 14 OD purified product. Characterization by ES-MS (neg. ion): 3596.65 ± 0.77 (M^+, $C_{129}H_{167}N_{57}O_{56}P_6^+$; calc. 3597.96).

5. *Synthesis of 5′-ACA TCA t(opg)gg tcg-hex-OH* (**2**; $n=2$, R = H). As described in *Exper. 3*, with HBTU (0.2M) as coupling reagent, with a pre-activation time of 30 min, and a total coupling time of 45 min. Yield of the crude product was 116 OD. Purification of crude product (48 OD) resulted in 6.8 OD purified product. Characterization by ES-MS (neg. ion): 3611.63 ± 0.1 (M^+; $C_{130}H_{169}N_{57}O_{56}P_6^+$; calc. 3611.99).

6. *Synthesis of 5′-ACA TCA t(obg)gg tcg-hex-OH* (**3**; $n=3$; R = H). As described in *Exper. 3*, with HBTU (0.2M) as coupling reagent a pre-activation time of 10 min, and a coupling time of 45 min. Yield of the crude product was 57 OD. Purification of crude product (30 OD) resulted in 5.8 OD purified product. Characterization by ES-MS (neg. ion): 3625.61 ± 0.09 (M^+, $C_{131}H_{171}N_{57}O_{56}P_6^+$; calc. 3626.01).

7. *Synthesis of 5′-ACA TCA t(opeg)gg tcg-hex-OH* (**4**; $n=4$; R = H). As described in *Exper. 3*, with HBTU (0.2M) as coupling reagent, with a pre-activation time of 15 min, and a total coupling time of 45 min. Yield of the crude product was 130 OD. Purification of crude product (56 OD) resulted in 19.9 OD purified product. Characterization by ES-MS (neg. ion): 3639.65 ± 0.07 (M^+, $C_{132}H_{173}N_{57}O_{56}P_6^+$; calc. 3640.04).

8. *Synthesis of 5′-ACA TCA t(oeg)gg tcg-hex-OH* (**5**; $n=1$; R = MeO). Synthesis was performed as described in *Sect. 3*, with HBTU (0.25M) as coupling reagent, a pre-activation time of 15 min, and a coupling reaction time of 17 min. Yield of the crude product was 78 OD. Purification of the crude product (39 OD) resulted in 11.1 OD purified product. Characterization by ES-MS (neg. ion): 3763.58 ± 0.07 (M^+, $C_{134}H_{162}N_{57}O_{62}P_6^+$; calc. 3764.09).

9. *Synthesis of 5′-ACA TCA t(opg)gg tcg-hex-OH* (**6**; $n=2$; R= MeO). As described in *Exper. 3*, with HBTU (0.25M) as coupling reagent, a pre-activation time of 15 min, and a total coupling time of 23 min. Yield of the crude product was 113 OD. Purification of the crude product (58 OD) resulted in 10.77 OD purified product. Characterization by EX-MS (neg. ion): 3777.62 ± 0.04 (M^+, $C_{135}H_{179}N_{57}O_{62}P_6^+$; calc. 3778.12).

10. *Synthesis of 5′-ACA TCA t(obg)gg tcg-hex-OH* (**7**; $n=3$; R= MeO). As described in *Exper. 3*, with HBTU (0.25M) as coupling reagent, a pre-activation time of 15 min, and a total coupling time of 30 min. The yield of the crude product was 116 OD. Purification of the crude product (57 OD) resulted in 17.7 OD purified product. Characterization by ES-MS (neg. ion): 3791.62 ± 0.03 (M^+, $C_{136}H_{181}N_{57}O_{62}P_6^+$; calc. 3792.15).

11. *Synthesis of 5′-ACA TCA t(opeg)gg tcg-hex-OH* (**8**; $n=4$; R= MeO). As described in *Exper. 3*, with HBTU (0.25M) as coupling reagent, a pre-activation time of 15 min, and a coupling time of 30 min. The yield of the crude product was 112 OD. Purification of the crude product (41 OD) resulted in 7.44 OD purified product. Characterization by ES-MS (neg. ion): 3805.93 ± 0.08 (M^+, $C_{137}H_{183}N_{57}O_{62}P_6^+$; calc. 3806.17).

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