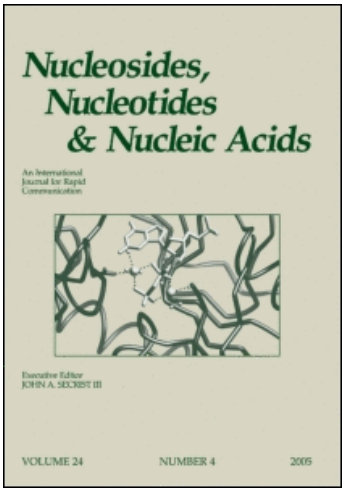

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SYNTHESIS OF DNA-(3')-PNA CHIMERAS WITH CONFORMATIONALLY RESTRICTED LINKERS BASED ON 4-HYDROXYPROLINE

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ABSTRACT: The synthesis and evaluation of DNA-(3')-PNA chimeras containing rigid linkers based on (*R/S*)-4-hydroxy-*D/L*-proline are described. It is shown that installment of the *trans*-*L*-linker using the tetrabutylammonium salt of (*R*)-4-(4,4'-dimethoxytrityloxy)-*N*-(thymin-1-yl)acetyl-*L*-proline (**2**) leads to a DNA-(3')-PNA chimera which hybridizes efficiently with complementary RNA.

INTRODUCTION

Since the advent of the Peptide Nucleic Acids (PNAs)[1], the synthesis of several chimeras comprising of PNA anchored to deoxyribose nucleic acid (DNA) has been reported.[2] For example, the conjugation of PNA to the 5'-terminus of DNA *via* a 5'-deoxy-5'-aminonucleoside gave access to PNA-(5')-DNA chimeras[3] (Fig. 1). The usefulness of PNA-(5')-DNA hybrids as tools in molecular biology was nicely illustrated by the application of these hybrids as PCR primers.[3c,4]

On the other hand, anchoring of PNA to the 3'-end of DNA using hydroxyethylglycinate (**1**) as the linker leads to DNA-(3')-PNA chimeras.[3b,5] An attractive feature of the latter type of hybrids is their inherent stability towards 3'-exonucleases, which are responsible for the major digestion of DNA *in vivo*.[6] In addition, these hybrids show improved cellular uptake[5b], are less prone to self-aggregation and are readily soluble in aqueous media. More interestingly, preliminary results indicated that

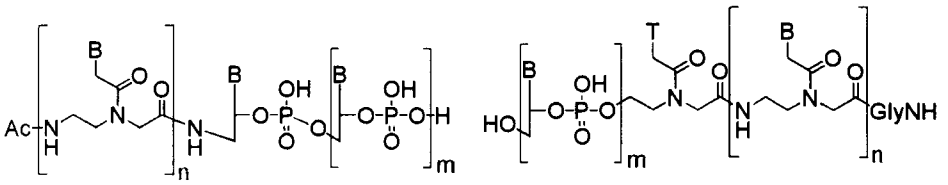


FIG. 1

DNA-(3')-PNAs activate RNase H mediated breakdown of complementary ribose nucleic acids (RNAs).[5b] The latter was substantiated by the finding that antisense DNA-(3')-PNA oligomers could inhibit the expression of the Luciferase gene in an RNase H dependent process.[3e] Unfortunately, the stability of DNA-(3')-PNAs with complementary (deoxy)oligonucleotides is significantly lower than for PNA•DNA and PNA•RNA duplexes.[3,5] The decrease in duplex stability indicates that the nature of the hydroxyethylglycine linker may oppose optimal hybridization of the chimera. Earlier studies revealed that replacement of the rather rigid 5'-amide linker in PNA-(5')-DNA chimeras (Fig. 1) by the more flexible 5'-phosphate had a negative effect on the hybridization properties.[3e] Based on this information, we reasoned that the duplex stability of DNA-(3')-PNA chimeras with complementary sequences could be increased by the application of a more conformationally restricted linker. Previous reports showed that hydroxyproline offers an attractive scaffold for the introduction of rigidity in PNA and related compounds.[8] The latter findings were an incentive to explore whether the replacement of the previously employed flexible *N*-[2-(4,4'-dimethoxytrityloxy)ethyl]-*N*-(thymin-1-ylacetyl)glycinate (**1**) by the individual rigid diastereomeric building blocks **2-5** (Fig. 2) would have a beneficial effect on the duplex stability with RNA or DNA.

As part of an ongoing program dealing with the design and synthesis of PNA-DNA chimeras for antisense purposes[3a,3e,7], we here report the synthesis of linker units **2-5**, their incorporation in DNA-(3')-PNA chimeras **32b-e** as well as the enzymatic and thermal stabilities of these new hybrids.

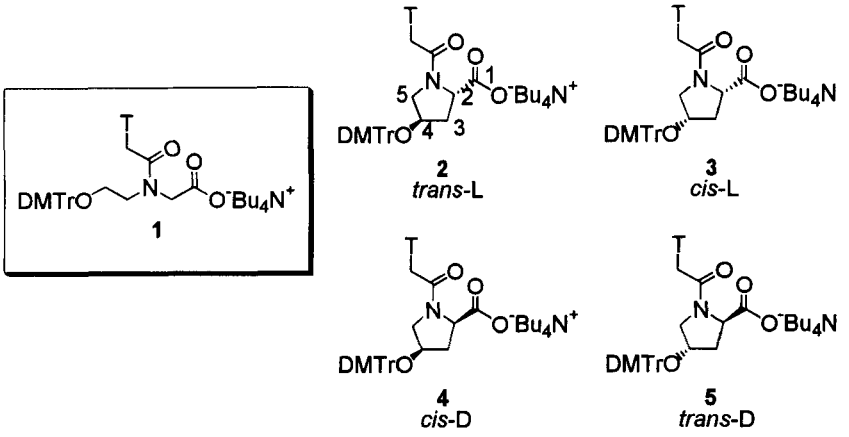
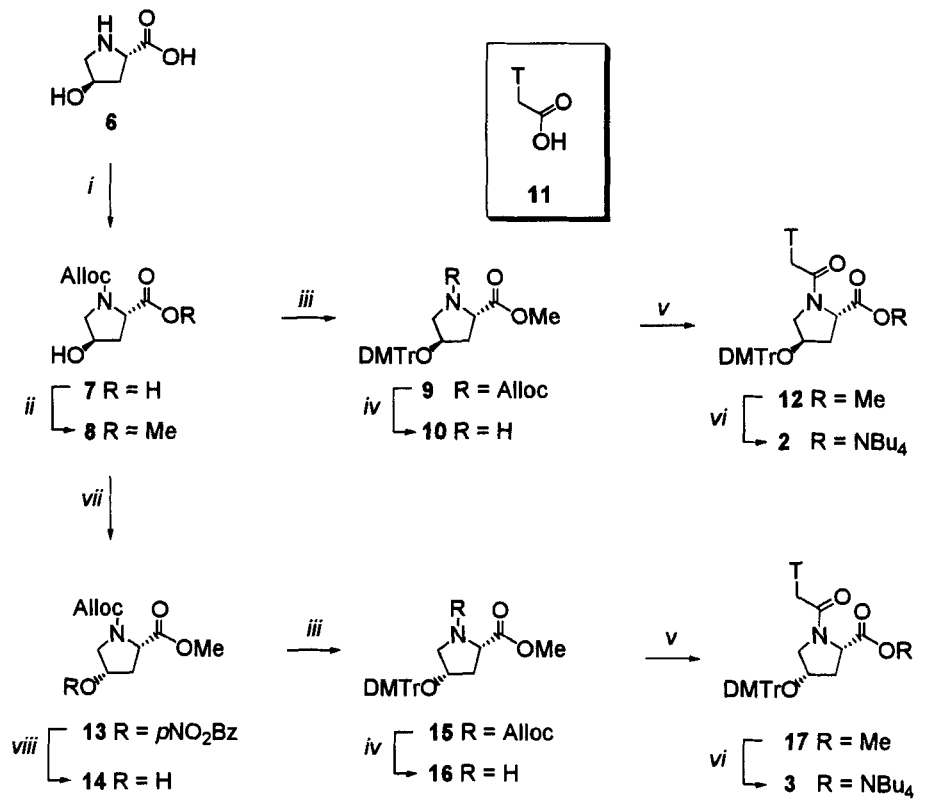


FIG. 2

RESULTS AND DISCUSSION

The synthesis of *trans-L*-linker **2** and *cis-L*-linker **3** can be achieved, as depicted in Scheme 1, starting from *trans*-4-hydroxy-*L*-proline **6**. Treatment of the α-amino function in **6** with allyloxycarbonyl chloride (AllocCl) under Schotten-Baumann conditions gave, after esterification[9] of the resulting carboxylic acid **7**, methyl prolinate **8**. Tritylation of **8** with dimethoxytrityl chloride (DMTrCl) at elevated temperature in pyridine containing catalytic amounts of tetrabutylammonium iodide (TBAI) and dimethylaminopyridine (DMAP) furnished fully protected **9**. Palladium(II) catalyzed hydrostannylation of **9** gave amine **10**. The thymine base was installed by coupling of (thymin-1-yl)acetic acid[10] (**11**) to the secondary amino group in backbone **10** using *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as the condensing agent. Treatment of the methyl ester in **12** with tetrabutylammonium hydroxide led to the isolation of homogeneous *trans*-hydroxy-*L*-proline derivative **2** in an overall yield of 31% over the six steps.

The synthesis of the *cis*-hydroxy-*L*-proline linker **3** started with inversion of the HO-4 group in **8** under modified Mitsunobu conditions (see Scheme 1).[11] Selective Zemplén

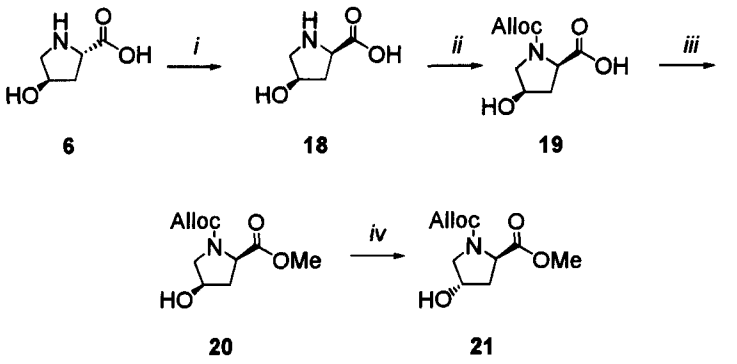


Reagents and conditions: *i)* AllocCl, $NaHCO_3$, Na_2CO_3, dioxane/H_2O, pH 9. *ii)* TMSCl, MeOH, 66% (two steps). *iii)* DMTrCl, TBAI, DMAP, pyridine, 50 °C, 78% (**9**), 85% (**15**). *iv)* Bu_3SnH, $PdCl_2(PPh_3)_2$, AcOH, CH_2Cl_2, 93% (**10**), 85% (**16**). *v)* **11**, DCC/HOBt, CH_2Cl_2, 81% (**12**), 92% (**17**). *vi)* $HONBu_4$, dioxane, H_2O, 80% (**2**), 69% (**3**). *vii)* DEAD, PPh_3, *p*-NO_2-BzOH, DMF, 66%. *viii)* KO*t*Bu, MeOH, 85%.

SCHEME 1

deacylation of the resulting *p*-nitrobenzoyl ester derivative **13** provided chirally pure **14**, which was subjected to the same sequence of reactions as described for the conversion of **8** into **2** [*i.e.* dimethoxytritylation (**14**→**15**), palladium catalyzed hydrostannylation (**15**→**16**), DCC/HOBt coupling (**16**→**17**) and saponification (**17**→**3**)] to provide monomer **3** in a yield of 46% based on **14**.

The preparation of hydroxy-*D*-proline linkers **4** and **5** commences with epimerization[12] of the C-2 position in **6** (Scheme 2). Thus, *trans*-hydroxy-*L*-proline **6** was acetylated by acetic anhydride in glacial acetic acid. The acylated product was heated

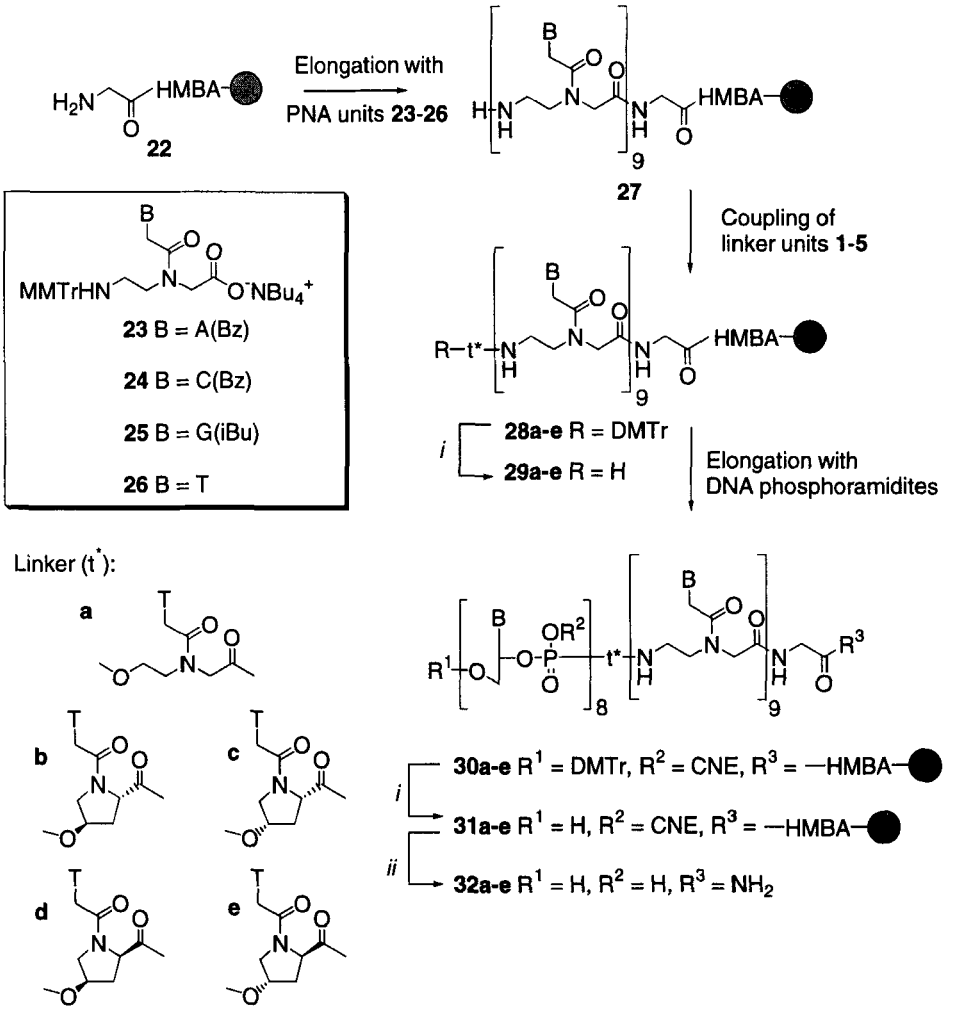


Reagents and conditions: *i)* 1. Ac_2O, AcOH, 2 N HCl. 2. Et_3N, 27%. *ii)* AllocCl, $NaHCO_3$, Na_2CO_3, dioxane/H_2O, pH 9. *iii)* TMSCl, MeOH, 63% (two steps). *iv)* 1. DEAD, PPh_3, *p*-NO_2-BzOH, DMF. 2. KO*t*Bu, MeOH, 54%.

SCHEME 2

under reflux in hydrochloric acid to give, after selective crystallization, the required *cis-D*-isomer **18**. Subjection of amino acid **18** to AllocCl under Schotten-Baumann conditions was followed by treatment of resulting **19** with trimethylsilyl chloride in methanol to give methyl ester **20**. Finally, inversion of the HO-4 under modified Mitsunobu conditions furnished *trans-D*-derivative **21**. Diastereomers **20** and **21** were then converted into *cis-D*- and *trans-D*-linkers **4** and **5** as described for their enantiomers **14** and **8**, respectively. The spectroscopic and spectrometric data (*i.e.* ^{1}H-NMR, ^{13}C-NMR, and mass) of *L*- and *D*-linker derivatives **2-3** and **4-5** were in full accord with the proposed structures. Furthermore, the specific optical rotations were of the same degree but opposite sign for each respective set of enantiomers.

Having the four diastereomeric linker molecules in hand, the stage was set for the assembly of DNA-(3')-PNA chimeras following a previously developed synthesis protocol.[7] Thus, anchoring of glycine to highly cross-linked polystyrene (PS) beads, functionalized with a 4-hydroxymethylbenzoic acid (HMBA) linker, resulted in the immobilized derivative **22** (Scheme 3). The PNA part of the chimera was assembled by sequential elongation of the amino-terminus of glycine derivative **22** with



Reagents and conditions: *i)* 3% TCA in DCE. *ii)* NH_3/MeOH, 50 °C, 15h.

SCHEME 3

monomethoxytrityl protected PNA monomers **23-26**[13] using 2-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent. After nine coupling cycles, the DNA-(3')-PNA linker was introduced by reaction of building blocks **1-5** with the amino group of resin bound PNA **27** to give **28a-e**. Stepwise elongation of the free hydroxyl group of the individual immobilized oligomers **29a-e**,

obtained after acidolysis of the DMTr group in **28a-e**, with commercially available phosphoramidites led to fully protected **30a-e**. In the final stage of the synthesis, detritylation of oligomer **30a-e** followed by ammonolysis (NH_3/MeOH)[14] of resulting **31a-e** gave, after purification by reversed-phase HPLC, homogeneous DNA-(3')-PNA chimeras **32a-e**.

The binding properties of the DNA-(3')-PNA chimeras **32a-e** with complementary DNA and RNA are recorded in Table 1. It can be seen that duplexation of the chimeras with DNA (entries 3-7) leads to less stable complexes in comparison with the corresponding DNA•DNA double helix (entry 1). A significant drop in thermal stability (12-14 °C) of the duplex is observed for hybrids **32c-e**. However, a relatively more stable duplex with DNA is formed in the case of chimera **32b** containing the *trans-L*-linker. Interestingly, the decrease in melting temperature (*i.e.* 3.6 °C) is comparable to the one observed for the chimera with flexible hydroxyethylglycine linker **32a** (3.2 °C, entry 3).

A similar tendency but higher thermal stability is observed (entries 3-7) for the duplexes of **32a-e** with RNA. Thus, hybrids **32c-e** give rise to slightly less stable duplex formation (entries 4-6) compared with the DNA•RNA duplex, whereas duplexation of **32a** and **32b** leads to helical structures with enhanced stability. Moreover, chimera **32b**, containing the *trans-L*-linker, proved to be comparable to the one based on the known flexible linker **32a** (*cf.* entries 3 and 7). As expected, the unmodified and modified chimeras **32a** and **32b** survived prolonged exposure to the 3'-exonucleolytic action of snake venom phosphodiesterase. HPLC analysis of the reaction mixtures revealed that the digestion of the DNA-(3')-PNA hybrids was negligible after 16h. For comparison, the corresponding native DNA oligomer was completely hydrolyzed within 1h under the reaction conditions.

CONCLUSIONS

4-Hydroxyproline has been successfully employed as the starting compound for the synthesis of conformationally restricted building blocks, suitable for the fully automated synthesis of DNA-(3')-PNA chimeras. The results presented in this paper indicate that further structural optimization of the linkers may lead to DNA-(3')-PNA chimeras with increased affinity for their target RNA sequences.

TABLE 1: Binding properties of DNA-(3')-PNAs with complementary DNA or RNA.

Entry	Duplex	Type		Tm with DNA (°C)	Tm with RNA (°C)
1	5'-ATT TCA TCT GCA ACT TCT 3'-TAA AGT AGA CGT TGA AGA	DNA DNA (RNA)		57.4	56.6
2	att tca tct gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	PNA DNA (RNA)		64.3 (+6.9)	73.1 (+16.5)
3	5'-ATT TCA TCt* gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	DNA-(3')-PNA DNA (RNA)	**32a**	54.2 (-3.2)	61.2 (+4.6)
4	5'-ATT TCA TCt* gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	DNA-(3')-PNA DNA (RNA)	**32c** (*cis-L*)	45.1 (-12.3)	53.3 (-3.3)
5	5'-ATT TCA TCt* gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	DNA-(3')-PNA DNA (RNA)	**32d** (*cis-D*)	44.9 (-12.5)	53.0 (-3.6)
6	5'-ATT TCA TCt* gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	DNA-(3')-PNA DNA (RNA)	**32e** (*trans-D*)	43.1 (-14.3)	49.9 (-6.7)
7	5'-ATT TCA TCt* gca act tct-$GlyNH_2$ 3'-TAA AGT AGA CGT TGA AGA	DNA-(3')-PNA DNA (RNA)	**32b** (*trans-L*)	53.8 (-3.6)	62.0 (+5.4)

Hybridization studies were performed in a phosphate buffered solution at pH 7, with a NaCl concentration of 100mM. Capital and small letters stand for DNA and PNA, respectively.

EXPERIMENTAL SECTION

^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-200 (operating at 200 MHz and 50.1 MHz, respectively). HH-COSY and CH-COSY spectra were recorded on a Bruker WM-300 (300 MHz and 75 MHz) or a Bruker DMX-600 (600 MHz and 150 MHz). ^{1}H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethyl silane as an internal standard. Mass spectra were recorded on a Finnigan MAT TSQ-70 or a Perkin Elmer Sciex API 165 equipped with an Electrospray Interface (ESI). Specific optical rotations were measured using a PROPOL automatic polarimeter at 20 °C with solutions in water or chloroform. Absorbance measurements were performed at 260 nm using a Perkin Elmer Lambda 12 UV/VIS Spectrometer equipped with a PTP-6 Peltier Temperature Programmer. Dichloromethane and pyridine were boiled under reflux with CaH_2 for 3 h, distilled and stored over molecular sieves (4 Å). Triethylamine was boiled under reflux with CaH_2 for 3 h and distilled. Ethyl acetate, toluene and light petroleum were distilled prior to use. 1,4-Dioxane (Baker, p.a.) and *N,N*-dimethylformamide (Baker, p.a.) were stored over molecular sieves (4 Å) and used as received. Methanol (Rathburn, HPLC grade) was stored over molecular sieves (3 Å) and used without further purification. Diisopropylethylamine (Biosolve, peptide grade) and acetonitrile (Rathburn, HPLC grade) were used as received. Acetic acid (Baker, p.a.), acetic anhydride (Baker, p.a.), allyl chloroformate (Fluka), chloro trimethylsilane (Aldrich), dichlorobis(triphenylphosphine)palladium(II) (Aldrich), *N,N'*-dicyclohexylcarbodiimide (Merck), 4,4'-dimethoxytrityl chloride (Acros), 4-(dimethylamino)pyridine (Merck), diethyl azodicarboxylate (Aldrich), 1-hydroxybenzotriazole (Acros), (*R*)-4-hydroxy-*L*-proline (Novabiochem), *p*-nitrobenzoic acid (Aldrich), tetrabutylammonium hydroxide (40% solution in water, Aldrich), tetrabutylammonium iodide (Acros), tributyltin hydride (Fluka) and triphenylphosphine (Acros) were used without further purification. A solution of SVP (*Crotalus atrox* Venom, 16 u/mL) was obtained from Pharmacia. Column chromatography was performed with silicagel 60, 230-400 mesh (Merck). TLC analysis was done on DC-fertigfolien (Schleicher & Schüll F1500, LS254) with detection by UV absorption (254 nm) and charring with a ninhydrine solution or a solution of ammonium molybdate (25 g/L) and ceric ammonium sulfate (10 g/L) in 10% aq. H_2SO_4. Reactions were run at ambient temperature, unless otherwise stated.

Methyl (*R*)-*N*-allyloxycarbonyl-4-hydroxy-*L*-prolinate (8): To a suspension of 10 mmol (1.31 g) (*R*)-4-hydroxy-*L*-proline (**6**) in dioxane (75 mL) and water (75 mL) containing Na_2CO_3 (20 mmol, 2.1 g) and $NaHCO_3$ (40 mmol, 3.35 g), was added 15 mmol (1.27 mL) allyl chloroformate. When TLC analysis indicated that the reaction was complete (2 h), water was added (50 mL) and the mixture was concentrated to a small volume (100 mL). The aqueous layer was washed twice with diethyl ether, neutralized with 2 N HCl and concentrated *in vacuo*. Traces of water were removed from thus obtained (*R*)-*N*-allyloxycarbonyl-4-hydroxy-*L*-proline (**7**) by coevaporation with dioxane. The carboxylic acid was dissolved in dry methanol (250 mL), TMSCl (65 mmol, 8.16 mL) was added and the solution was allowed to stir for 48 hours. The solvents were removed *in vacuo* and the methyl ester was dissolved in ethyl acetate. The organic layer was washed with water, 1 N $NaHCO_3$ and brine, dried over $MgSO_4$, filtered and evaporated. Yield: 1.51 g (6.61 mmol, 66% over 2 steps). ^{1}H NMR (CDCl3): δ 5.89, m, 1H, CH allyl; 5.24 m, 2H, CH_2 allyl; 4.63, d, 2H, CH_2 allyl, J=5.1 Hz; 4.57, m, 2 H, H-4',

H-2'; 3.75 (mi), 3.72 (ma), s, 3H, OMe; 3.63, m, 2H, H-5'a,b; 2.33, m, 1H, H-3'a; 2.07, m, 1H, H-3'b. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 173.1 C_q ester; 154.8 (ma), 154.4 (mi) C_q alloc; 132.4 (mi), 132.1 (ma) CH allyl; 117.3 (ma), 117.0 (mi) CH_2 allyl; 69.4 (ma), 68.7 (mi) CH; 65.9 CH_2 allyl; 57.7 (ma), 57.5 (mi) CH; 54.9 (mi), 54.3 (ma) C-5'; 52.1 OMe; 38.8 (mi), 38.1 (ma) C-3'.

Methyl (*R*)-*N*-allyloxycarbonyl-4-(4,4'-dimethoxytrityloxy)-*L*-prolinate (9): 0.52 g (2.28 mmol) **8** was dissolved in pyridine (15 mL) containing catalytic amounts of TBAI and DMAP. 1.16 g (3.5 mmol) of DMTCl was added and the solution was left at 50 °C for 16 h. The reaction was quenched by addition of methanol and the solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate (25 mL) and washed with water, 1 N $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated. Purification by column chromatography (25-30% ethyl acetate in light petroleum, containing 0.5% NEt_3) yielded 1.03 g (1.78 mmol, 78%) of **5a**. ^1H NMR ($CDCl_3$): δ 7.34-6.80, m, 13H, CH arom. (DMT); 5.87, m, 1H, CH allyl; 5.23, m, 2H, CH_2 allyl; 4.56, m, 2H, CH_2 allyl; 4.39, m, 1H, H-4'; 4.25, m, 1H, H-2'; 3.79, s, 6H, OMe (DMT); 3.65 (ma), 3.61 (mi), s, 3H, OMe; 3.27, m, 2H, H-5'; 1.91, m, 1H, H-3'a; 1.53, m, 1H, H-3'b. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 172.3 (ma), 172.1 (mi) C_q ester; 158.2 C_q arom. (DMT); 154.0 (ma), 153.3 (mi) C_q alloc; 144.8 C_q arom. (DMT); 135.7 C_q arom. (DMT); 132.2, 129.4, 127.4, 126.4 CH arom. (DMT); 116.3 CH_2 allyl; 112.7 CH arom. (DMT); 86.3 C_q (DMT); 71.2 (ma), 70.5 (mi) CH; 65.1 CH_2 allyl; 57.3 (ma), 57.1 (mi) CH; 54.5 OMe (DMT); 52.0 (ma), 51.4 (mi) C-5'; 51.3 OMe; 36.7 (mi), 35.9 (ma) C-3'.

Methyl (*R*)-4-(4,4'-dimethoxytrityloxy)-*L*-prolinate (10): 1.9 g (3.6 mmol) Alloc protected **9** was dissolved in 25 mL dichloromethane. To this solution was added 0.38 mL (6.75 mmol) glacial acetic acid, 1.79 mL (6.75 mmol) Bu_3SnH and a catalytic amount of $Pd(PPh_3)_2Cl_2$. After 30 min TLC (1/4 ethyl acetate/light petroleum v/v) indicated that the deprotection was complete. NEt_3 was added (1.5 mL) and the dichloromethane was removed *in vacuo*. The residue was dissolved in ethyl acetate (75 mL) and washed with water, 1 N $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$ to furnish 1.47 g (3.36 mmol, 93%) **10** after column chromatography (1/1 ethyl acetate/light petroleum v/v with 0.5% NEt_3). ^1H NMR ($CDCl_3$): δ 7.42-6.80, m, 13 H, CH arom.(DMT); 4.14, m, 1H, CH; 3.85, m, 1H, CH; 3.79, s, 6H, OMe (DMT); 3.66, s, 3H, OMe; 2.60, AB, 2H, H-5'a,b; 1.87, m, 1H, H-3'a; 1.67, m, 1H, H-3'b. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ174.3 C_q ester; 158.0 C_q arom. (DMT); 145.2 C_q arom. (DMT); 136.2 C_q arom. (DMT); 129.6, 127.6, 127.3, 126.2, 112.6 CH arom. DMT; 86.1 C_q DMT; 73.7 CH; 59.7 CH; 54.5 OMe DMT; 52.9 C-5'; 51.3 OMe; 37.4 C-3'.

Methyl (*R*)-4-(4,4'-dimethoxytrityloxy)-*N*-(thymin-1-yl)acetyl-*L*-prolinate (12): Amine **10** (1.47 g, 3.4 mmol) was dissolved in 20 mL DMF containing 0.68 g (3.7 mmol) (thymin-1-yl)acetic acid (**11**). The solution was subsequently treated with 0.5 g (3.7 mmol) HOBt and 0.83 g (4.0 mmol) DCC. The resulting suspension was allowed to stand overnight, after which the precipitated DCU was removed through filtration over Hyflo. DMF was evaporated and the residues were dissolved in 50 mL ethyl acetate and washed with water, 1 N $NaHCO_3$ and brine. After drying over $MgSO_4$, filtration and concentration under reduced pressure, column chromatography (3/1 ethyl acetate/light petroleum v/v, containing 0.5% NEt_3) provided 1.38 g (2.3 mmol, 64%) amide **12**. ^1H NMR ($CDCl_3$): δ 7.46-6.80, m, 14 H, CH arom. (DMT), CH arom. T; 4.59, m, 1H, CH;

4.36, m, 2H, CH_2; 4.07, m, 1H, CH; 3.79, s, 6H, OMe (DMT); 3.66, s, 3H, OMe; 3.13, m, 1H, H-5'a; 2.96, m, 1H, H-5'b; 2.15, m, 1H, H-3'a; 1.88, s, 3H, CH_3 T; 1.78, m, 1H, H-3'b. ^{13}C {^1H} NMR ($CDCl_3$): δ 171.6 (ma), 171.3 (mi) C_q ester; 165.4 (mi), 164.9 (ma) T C-2; 164.2 C_q amide; 158.2 C_q arom. (DMT); 150.8 T C-4; 144.6 C_q arom. (DMT); 140.6 T C-6; 135.6 C_q arom. (DMT); 128.1, 127.9, 127.5, 126.6, 112.9 CH arom. DMT; 109.7 T C-5; 86.5 C_q DMT; 71.6 CH; 59.8 CH_2; 57.2 CH; 54.7 OMe DMT; 52.3 OMe; 51.8 C-5'; 47.5 CH_2; 35.1 C-3'; 11.7 T CH_3.

Tetrabutylammonium (*R*)-4-(4,4'-dimethoxytrityloxy)-*N*-(thymin-1-yl)acetyl-*L*-prolinate (2): Addition at 10 °C of 40 mL of a 40% $HONBu_4$ solution to 0.69 g (1.13 mmol) methyl ester **12** was followed after 2 min by dilution with 100 mL water. The aqueous layer was extracted with dichloromethane (2 x 150 mL) and the combined dichloromethane layers were washed with water until neutral (10 x 150 mL). After drying over $MgSO_4$ and filtration, the target compound **2** was obtained through evaporation of the organic solvent. Yield: 0.79 g (0.90 mmol, 80%). ^1H (COSY) NMR ($CDCl_3$): δ 7.44-6.77, m, 14H, CH arom. (DMT), CH arom. T; 4.78, d, 1H, H-6'a (J= 6.9 Hz); 4.31, m, 3H, H-4', H-2', H-6'; 3.75, s, 6H, OMe (DMT); 3.20, m, 10H, CH_2 TBA, H-5'; 2.21, m, 2H, H-3'; 1.80, s, 3H, CH_3 T; 1.59, m, 8H, CH_2 TBA; 1.36, q, 8H, CH_2 TBA (J=7.2Hz); 0.97, t, 12H, CH_3 TBA (J=7.3Hz). ^{13}C (CH-COSY) NMR ($CDCl_3$): δ 174.0 Cq carboxylate,165.2 T C-2; 164.7 C_q amide; 158.1 C_q DMT; 151.0 T C-4; 145.1 C_q DMT; 142.0 T C-6; 136.3 C_q DMT; 129.5, 127.6, 127.3, 126.2,112.7 CH arom. DMT; 108.8 T C-5; 85.9 C_q DMT; 70.3 CH; 60.1 CH; 58.0 CH_2 TBA; 54.6 OMe DMT; 52.3 C-5'; 47.5 CH_2; 38.3 C-3'; 23.3 CH_2 TBA; 19.2 CH_2 TBA; 13.2 CH_3 TBA; 11.7 T CH_3. Mass spectroscopy (ESI): calculated: 599, found: m/z=598 $(M-H)^-$. $[\alpha]_D$ = -36.8 ° (c=1, $CHCl_3$).

Methyl (*S*)-*N*-allyloxycarbonyl-4-hydroxy-*L*-prolinate (14): 1.24 g (5.4 mmol) **8** was dissolved in 50 mL DMF and cooled to 0°C. 2.83 g (10.8 mmol) PPh_3 in 5 mL DMF was added dropwise, followed by the dropwise addition of a solution of *p*-nitrobenzoic acid (1.84 g, 10.8 mmol) and 1.6 mL DEAD (9.5 mmol) in 10 mL DMF. The reaction was allowed to proceed for 16 h and after the usual work-up, column chromatography (50 - 70% ethyl acetate in light petroleum) yielded 1.34 g (3.5 mmol, 66%) of the (*R*)-*p*-nitrobenzoic ester (**13**). The ester was dissolved in 100 mL MeOH containing a catalytic amount (10 mg) of KO*t*Bu. Deacylation was established after 2 h and the reaction mixture was neutralized by the addition of acetic acid. The solvents were removed *in vacuo* and the residues were dissolved in 50 mL ethyl acetate and washed with water (2 x 30 mL), $NaHCO_3$ (1 N, 2x 30 mL) and brine (30 mL). Drying over $MgSO_4$ and removal of the solvents was followed by column chromatography (ethyl acetate) for purification. Yield: 0.68 g (2.97 mmol, 85% over the last step, 55% from **8**). ^1H NMR ($CDCl_3$): δ 5.90, m, 1H, CH allyl; 5.27, m, 2H, CH_2 allyl; 4.61, d, 2H, CH_2 allyl, J=5.1 Hz; 4.41, m, 2H, H-2', H-4'; 3.79 (ma), 3.76 (mi), s, 3H, OMe; 3.64, m, 2H, H-5'a,b; 2.34, m, 1H, H-3'a; 2.13, m, 1H, H-3'b. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 172.5 C_q ester; 154.0 (ma), 153.6 (mi) C_q alloc; 131.9 CH allyl; 116.5 (ma), 116.2 (mi) CH_2 allyl; 69.1 (ma), 68.2 (mi) CH; 65.1 CH_2 allyl; 57.1 (ma), 56.8 (mi) CH; 54.2 (mi), 53.8 (ma) C-5'; 51.5 OMe; 37.9 (mi), 37.0 (ma) C-3'.

Methyl (*S*)-*N*-allyloxycarbonyl-4-(4,4'-dimethoxytrityloxy)-*L*-prolinate (15): Following the same procedure as described for the synthesis of compound **9**, 0.97 g (4.25

mmol) of **14** and 2.2 g (6.4 mmol) of DMTCl yielded 2.09 g (3.6 mmol, 85%) **15**. ^{1}H NMR ($CDCl_3$): δ 7.44-6.81, m, 13H, CH arom. DMT; 5.88, m, 1H, CH allyl; 5.20, m, 2H, CH_2 allyl; 4.56, d, 2H, CH_2, allyl, J=5.1 Hz; 4.17, m, 2H, H-2', H-4'; 3.79, s, 6H, OMe (DMT); 3.71 (mi), 3.69 (ma), s, 3H, OMe; 3.43, m, 1H, H-5'a; 3.27, d, 1H, H-5'b, J=5.1; 1.82, m, 1H, H-3'a; 1.61, m, 1H, H-3'b. ^{13}C {^{1}H} NMR ($CDCl_3$): δ 171.8 (mi), 171.7 (ma) C_q ester; 158.2 (mi), 158.1 (ma) C_q arom. (DMT); 154.0 (mi), 153.6 (ma) C_q alloc; 144.8 C_q arom. (DMT); 135.9 C_q arom. (DMT); 129.7, 128.7, 127.2, 126.5 CH arom. (DMT); 116.4 CH_2 allyl; 112.8 CH arom. (DMT); 86.6 C_q DMT; 71.6 (mi), 70.8 (ma) CH; 65.2 CH_2 allyl; 57.1 CH; 54.6 OMe DMT; 52.7 (ma), 52.0 (mi) C-5'; 51.6 OMe; 36.8 (ma), 35.9 (mi) C-3'.

Methyl (*S*)-4-(4,4'-dimethoxytrityloxy)-*L*-prolinate (16): Removal of the Alloc group was established as described for compound **10**. Yield: 1.44 g (3.06 mmol, 85%) starting from 2.09 g (3.6 mmol) **15**, 0.41 mL (7.2 mmol) glacial acetic acid and 1.94 mL (7.2 mmol) Bu_3SnH. ^{1}H NMR ($CDCl_3$): δ 7.44-6.78, m, 13 H, CH arom. (DMT); 4.11, m, 1H, CH; 3.78, s, 6H, OMe (DMT); 3.72, s, 3H, OMe; 3.59, m, 1H, CH; 2.58, m, 2H, H-5'a,b; 1.94, m, 1H, H-3'a; 1.67, m, 1H, H-3'b. ^{13}C {^{1}H} NMR ($CDCl_3$): δ 175.1 C_q ester; 158.3 C_q arom. (DMT); 145.5 C_q arom. (DMT); 136.6 C_q arom. (DMT); 129.9, 128.1, 127.6, 126.6, 112.9 CH arom. (DMT); 86.5 C_q DMT; 73.8 CH; 58.5 CH; 54.8 OMe DMT; 53.2 C-5'; 51.7 OMe; 37.4 C-3'.

Methyl (*S*)-4-(4,4'-dimethoxytrityloxy)-*N*-(thymin-1-yl)acetyl-*L*-prolinate (17): Amide bond formation between 0.73 g (1.55 mmol) amine **16** and 0.3 g (1.6 mmol) (thymin-1-yl)acetic acid (**11**) using 0.22 g (1.6 mmol) HOBt and 0.37 g (1.8 mmol) DCC furnished 0.86 g (1.43 mmol, 92%) of the title compound following a similar protocol as described for **12**. ^{1}H NMR ($CDCl_3$): δ 7.43-6.80, m, 14H, CH arom. (DMT), CH arom. T; 4.78-4.32, m, 2H, CH_2; 4.23, m, 1H, CH; 3.97, m, 1H, CH; 3.79, s, 6H, OMe (DMT); 3.72 (ma). 3.70 (mi), s, 3H, OMe; 3.49, m, 1H, H5'a; 3.17, m, 1H, H5'b; 1.91 (mi), 1.88 (ma), s, 3H, CH_3 T; 1.86, m, 2H, H-3'ab; 1.74, s, 3H, CH_3 T. ^{13}C {^{1}H} NMR ($CDCl_3$): δ 171.8 (mi), 171.3 (ma) C_q ester; 167.1 (ma), 166.1 (mi) T C-2, 164.6 C_q amide; 158.6 C_q arom. (DMT); 151.5 (mi), 151.3 (ma) T C-4; 144.8 C_q arom. (DMT); 141.3 (mi), 141.1 (ma) T C-6; 136.0 C_q arom. (DMT); 129.9, 127.8, 127.0, 113.2 CH arom. (DMT); 110.2 T C-5; 87.2 C_q DMT; 72.2 (ma), 70.3 (mi) CH; 58.0 (mi), 57.3 (ma) CH; 55.1 OMe DMT; 52.6 (mi), 52.3 (ma) OMe; 52.0 C-5'; 48.4 (mi), 48.0 (ma) CH_2; 37.3 (mi), 35.4 (ma) C-3'; 10.8 T CH_3.

Tetrabutylammonium (*S*)-4-(4,4'-dimethoxytrityloxy)-*N*-(thymin-1-yl)acetyl-*L*-prolinate (3): 0.83 g (0.99 mmol, 69%) of the title compound was obtained starting from 0.88 g (1.43 mmol) of the methyl ester as described for **2**. ^{1}H (COSY) NMR ($CDCl_3$): δ 7.44-6.78, m, 14H, CH arom. (DMT), CH arom. T; 4.63, AB, 2H, CH_2; 4.05, m, 2H, H-4', H-2'; 3.76, s, 6H, OMe (DMT); 3.52, m, 1H, H-5'a; 3.31, m, 8H, CH_2 TBA; 3.10, m, 1H, H-5'b; 2.05, m, 2H, H-3'a,b; 1.82, s, 3H, CH_3 T; 1.64, m, 8H, CH_2 TBA; 1.43, q, 8H, CH_2 TBA, J=7.4Hz; 1.01, t, 12H, CH_3 TBA, J=7.3 Hz. ^{13}C (CH-COSY) NMR ($CDCl_3$): δ 173.9 Cq carboxylate;165.6 T C-2; 164.5 C_q amide; 158.0 C_q DMT; 150.9 T C-4; 145.2 C_q DMT; 142.1 T C-6; 136.5 C_q DMT; 128.6, 127.7, 127.4, 126.3, 112.7 CH arom. DMT; 108.8 T C-5; 85.9 C_q DMT; 70.1 CH; 59.6 CH; 58.2 CH_2 TBA; 54.7 OMe DMT; 50.8 C-5'; 47.6 CH_2; 37.8 C-3'; 23.5 CH_2 TBA; 19.3 CH_2 TBA; 13.3 CH_3 TBA; 11.7 T CH_3. Mass spectroscopy (ESI): calculated: 599, found: m/z=598 $(M-H)^-$. $[\alpha]_D$ = -1.4 ° (c=1, $CHCl_3$).

(*R*)-4-hydroxy-*D*-proline (18): A solution of 50 mL acetic anhydride in 100 mL glacial acetic acid and 6.65 g **6** was heated to reflux temperature and the solution was held at reflux for 5.5 h. The solvent was removed *in vacuo*, giving a thick oil which was dissolved in 100 mL 2 N HCl and heated to reflux temperature for 3 hours. The solution was treated with charcoal and filtered while hot. After removal of the solvent under reduced pressure, the mixture of the HCl-salt of *D*- and *L*-4-hydroxyproline was dissolved in 2 mL water and 1 mL triethylamine and **18** was obtained through selective crystallization overnight at -20° C from absolute ethanol. Yield 1.75 g (13.4 mmol, 27%). ^{1}H NMR (D_2O): δ 4.58, bs 1H, H'-4; 4.22, dd, 1H, H-2', $J_{2'-3'a}$=4.5 Hz, $J_{2'-3'b}$=10.8 Hz.; 3.39, m, 2H, H 5'a,b; 2.52, m, 1H, H-3'a; 2.27, m, 1H, H-3'a. ^{13}C{^{1}H} NMR (D_2O): δ 173.5 C_q acid; 69.9 CH; 60.4 CH; 53.7 C-5'; 37.9 C-3'. $[\alpha]_D$ = +63.6 ° (c=1, H_2O), lit. $[\alpha]_D$ = +59.5 ° (c=2, H_2O).

Methyl (*R*)-*N*-allyloxycarbonyl-4-hydroxy-D-prolinate (20): Following a similar protocol as reported for **8**, 1.51 g (6.61 mmol, 63% over two steps) of the title compound was obtained. ^{1}H NMR (CDCl3): δ 5.90, m, 1H, CH allyl; 5.26 m, 2H, CH_2 allyl; 4.61, d, 2H, CH_2 allyl, J=5.1 Hz; 4.43, m, 2 H, H-4', H-2'; 3.78 (ma), 3.76 (mi), s, 3H, OMe; 3.64, m, 2H, H-5'a,b; 2.34, m, 1H, H-3'a; 2.12, m, 1H, H-3'b. ^{13}C{^{1}H} NMR ($CDCl_3$): δ 172.3 C_q ester; 154.0 (mi), 154.5 (ma) C_q alloc; 131.9 CH allyl; 116.4 (ma), 116.0 (mi) CH_2 allyl; 69.0 (mi), 68.0 (ma) CH; 65.9 CH_2 allyl; 57.0 (ma), 56.8 (mi) CH; 54.1 (mi), 53.7 (ma) C-5'; 51.4 OMe; 37.9 (mi), 37.0 (ma) C-3'.

Methyl (*S*)-*N*-allyloxycarbonyl-4-hydroxy-D-prolinate (21): Inversion of configuration at the 4-postion was established as described for **14**, to give 0.52 g (54%) of the target compound. All spectroscopic and spectrometric properties were identical to those described for compound **8**.

General procedure for the oligomer synthesis: solid phase syntheses were performed on a Pharmacia Gene Assembler using highly cross-linked polystyrene beads as the solid support (loading: 26-28 μmol/g) on a 1 μmol scale. The support was functionalized with a glycine moiety via a *p*-hydroxymethylbenzoic acid linker. Assembly of the PNA part was established using solutions of 0.3 M of monomers **23-26**, 0.3 M DiPEA and 0.3 M HATU in acetonitrile/dimethylformamide (1/1, v/v). Prior to coupling, the monomers were pre-activated for 1 min by mixing equal amounts of the PNA monomer (15 equiv per μmol support), HATU and DiPEA solutions. The protocol for one PNA chain extension cycle consisted of (1) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL; (2) coupling: PNA + HATU + DiPEA in acetonitrile/dimethylformamide (1/1, v/v), 15 min; (3) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL, acetonitrile, 2.5 mL; (4) capping: Ac_2O/lutidine/*N*-methylimidazole/tetrahydrofuran (1/1/1/7, v/v/v/v), 2.0 mL; (5) wash: acetonitrile, 2.5 mL, dichloromethane, 3.5 mL; (6) detritylation: 2% trichloroacetic acid in dichloromethane, 3 min; (7) wash: dichloromethane, 2.5 mL, acetonitrile, 5 mL. In the final step of the PNA synthesis, linkers **a-e** were attached to the terminal aminogroup by coupling of monomers **1-5**, respectively. The introduction of the phosphate bonds was carried out with 15 equiv of commercially available cyanoethyl protected DNA phosphoramidites using 5-(*ortho*-nitrophenyl)tetrazole (8 equiv) as the activator. Standard DNA capping, washing, oxidation and detritylation cycles were used. Coupling yields were gauged spectrophotometrically (254 nm) by the absorption of the released trityl cation after each deprotection step. After the last elongation step, the oligomers were cleaved from the support with concomitant deprotection of the phosphate

groups and exocyclic amino groups by treatment with methanolic ammonia (1.5 mL) at 50 °C for 16 h. RP-HPLC purification and analysis were carried out on a Jasco HPLC system equipped with a LiChrospher 100 RP-18 endcapped column (10.0 x 250 mm and 4.0 x 250 mm, respectively). Gradient elution was performed at 40 °C by building up a gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 1/1, v/v) with a flow rate of 1.0 mL/min or 5.0 mL/min for analysis and purification, respectively.

Mass spectrometry analysis of the oligomers: The identity of the oligomers was confirmed by MALDI-TOF mass spectrometry (negative mode) at Eurogentec S.A. (Belgium). Results: **32a**: m/z = 5146.3 (calculated 5156.2 for $[C_{187}H_{238}N_{77}O_{84}P_8]^-$); **32b**: m/z = 5168.9 (calculated 5168.2 for $[C_{188}H_{238}N_{77}O_{84}P_8]^-$); **32c**: m/z = 5169.1 (calculated 5168.2 for $[C_{188}H_{238}N_{77}O_{84}P_8]^-$); **32d**: m/z = 5165.2 (calculated 5168.2 for $[C_{188}H_{238}N_{77}O_{84}P_8]^-$); **32e**: m/z = 5164.0 (calculated 5168.2 for $[C_{188}H_{238}N_{77}O_{84}P_8]^-$).

Thermal melting studies: Melting temperatures (Tm) were determined with equimolar mixtures of the oligonucleotide and complementary RNA at a concentration of 3 μM, in a 10 mM phosphate buffer, pH=7.0, containing NaCl (100 mM) and EDTA (0.1 mM). Prior to recording the melting profile, the solutions were heated to 90 °C for several minutes and then cooled to 5 °C over 2.0 h. The mixtures were kept at 5 °C for 16 h and heated to 80 °C at a rate of 0.5 °C per minute while the A_{260} was recorded against temperature. The Tm values were determined as the maxima of the first-derivative plots of absorbance versus temperature.

Enzymatic stability studies: 0.6 OD of the oligomer was dissolved in 2.0 mL 100 mM Tris.HCl buffer, pH=8.6, containing 100 mM NaCl and 14 mM $MgCl_2$. Upon addition of 1 unit of snake venom phosphodiesterase (62 μl of a solution in water) the enzymatic degradation was monitored at 20 °C using a Biocad Vision reversed-phase HPLC system equipped with a LiChrospher 100 RP-18 endcapped column. Elution was carried out with a gradient of acetonitrile (0-20%) in 50 mM triethylammonium acetate. Analysis was done with aliquots taken from the reaction mixtures after 1 h or 16 h.

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REFERENCES AND NOTES

1. Nielsen, P.E.; Egholm, M.; Berg, R.H.; Buchardt, O. *Science*, **1991**, *254*, 1497-1500.

2. For a recent review on PNA-DNA chimeras, see: Uhlmann, E. *Biol. Chem.*, **1998**, *379*, 1045-1052.
3. a) van der Laan, A.C.; Meeuwenoord, N.J.; Kuyl-Yeheskiely, E.; Oosting, R.S.; Brands, R.; van Boom, J.H. *Recl. Trav. Chim. Pays-Bas*, **1995**, *114*, 295-297. b) Bergmann, F.; Bannwarth, W.; Tam, S. *Tetrahedron Lett.*, **1995**, *36*, 6823-6826. c) Stetsenko, D.A; Lubyako, E.N.; Potapov, V.K. Azhikina, T.L.; Sverdlov, E.D. *Tetrahedron Lett.*, **1996**, *37*, 3571-3574. d) Finn, P.J.; Gibson, N.J.; Fallon, R.; Hamilton, A.; Brown, T. *Nucl. Acids Res.*, **1996**, *24*, 3357-3363. e) van der Laan, A.C.; Havenaar, P.; Oosting, R.S.; Kuyl-Yeheskiely, E.; Uhlmann, E.; van Boom, J.H. *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 663-668.
4. Misra, H.S.; Pandey, P.K.; Modak, M.J.; Vinayak, R.; Pandey, V.N. *Biochemistry*, **1998**, *37*, 1917-1925.
5. a) Petersen, K.H.; Jensen, D.K.; Egholm, M.; Nielsen, P.E.; Buchardt, O. *Bioorg. Med. Chem. Lett.*, **1995**, *5*, 1119-1124. b) Uhlmann, E.; Will, D.W.; Breipohl, G.; Langner, D.; Ryte, A. *Angew. Chem. Int. Ed.. Engl.*, **1996**, *35*, 2632-2635.
6. Uhlmann, E.; Peyman, A. *Chem. Rev.*, **1990**, *90*, 543-584.
7. a) van der Laan, A.C.; Brill, R.; Kruimelis, R.G.; Kuyl-Yehskiely, E.; van Boom, J.H.; Andrus, A.; Vinayak, R. *Tetrahedron Lett.*, **1997**, *38*, 2249-2252. b) Vinayak, R.; van der Laan, A.C.; Brill, R.; Otteson, K.; Andrus, A.; Kuyl-Yeheskiely, E.; van Boom, J.H. *Nucleosides and Nucleotides*, **1997**, *16*, 1653-1656.
8. a) Gangamani, B.P.; Kumar, V.A.; Ganesh, K.N. *Tetrahedron*, **1996**, *52*, 15017-150130. b) Jordan, S.; Schwemler, C.; Kosch, W.; Kretschmer, A.; Schwenner, E.; Stropp, U.; Mielke, B. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 681-686. c) Jordan, S.; Schwemler, C.; Kosch, W.; Kretschmer, A.; Stropp, U.; Schwenner, E.; Mielke, B. *ibid.*, **1997**, *7*, 687-690. d) Lowe, G.; Vilaivan, T. *J. Chem. Soc., Perkin Trans. 1*, **1997**, 539-546. e) Lowe, G.; Vilaivan, T. *ibid.*, **1997**, 547-554.
9. Brook, M.A.; Chan, T.H. *Synthesis*, **1983**, 201-203.
10. Jones, A.S.; Lewis, P.; Withers, S.F. *Tetrahedron*, **1973**, *29*, 2293-2296.
11. a) Mitsunobu, O. *Synthesis*, **1981**, 1-27. b) Martin, S.F.; Dodge, J.A. *Tetrahedron Lett.*, **1991**, *32*, 3017-3020.
12. Robinson, D.S.; Greenstein, J.P. *J. Biol. Chem.*, **1952**, *195*, 383-388.
13. a) Will, D.W.; Breipohl, G.; Langner, D.; Knolle, J.; Uhlmann, E. *Tetrahedron*, **1995**, *51*, 12069-12082. b) Breipohl, G.; Will, D.W.; Peyman, A.; Uhlmann, E. *Tetrahedron*, **1997**, *53*, 14671-14686.

14. Ammonolysis using NH_4OH instead of methanolic ammonia results in the undesirable formation of mixtures of C-terminal carboxylate and amide.

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