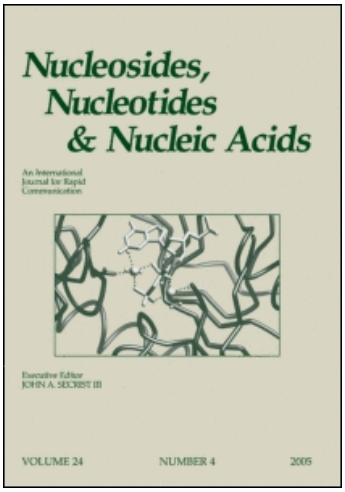

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POLYESTER AND N-METHYL ANALOGUES OF PEPTIDE NUCLEIC ACIDS: SYNTHESIS AND HYBRIDIZATION PROPERTIES

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ABSTRACT: The synthesis of a set of homo-thymine analogues of peptide nucleic acids containing ester or N-methylamide linkages as well as analogues derived from *trans*-4-hydroxy-L-proline and their hybrids has been accomplished, and their binding affinity to complementary DNA and RNA strands was evaluated.

Introduction

Among DNA analogues developed as potential antisense and antigene reagents and diagnostic tools, peptide nucleic acids (PNAs) are very promising compounds due to their excellent binding properties and enzymatic stability.[1] Recently, we have undertaken the investigations on the design and synthesis of PNA-related DNA mimics with the aim to improve their physico-chemical and biological properties, particularly water solubility and cellular uptake.[2,3] Mimic oligomers representing phosphonate analogues of PNAs (pPNAs) and PNA-pPNA chimeras containing the four natural nucleobases have been obtained and their properties were examined (Fig. 1A). It was found that, along with good water solubility, they form stable complexes with complementary DNA and RNA fragments, and, particularly, PNA-pPNA hetero-oligomers were found to be promising for further evaluation as potential antisense and antigene agents.[4-6]

Earlier, the oligonucleotide analogues derived from 4-aminoproline[7,8] and 4-hydroxy-N-acetylprolinol[9] were described, and it was shown that the incorporation of *trans*-4-amino-L-proline derivatives into PNA chains resulted in the improvement of their base-

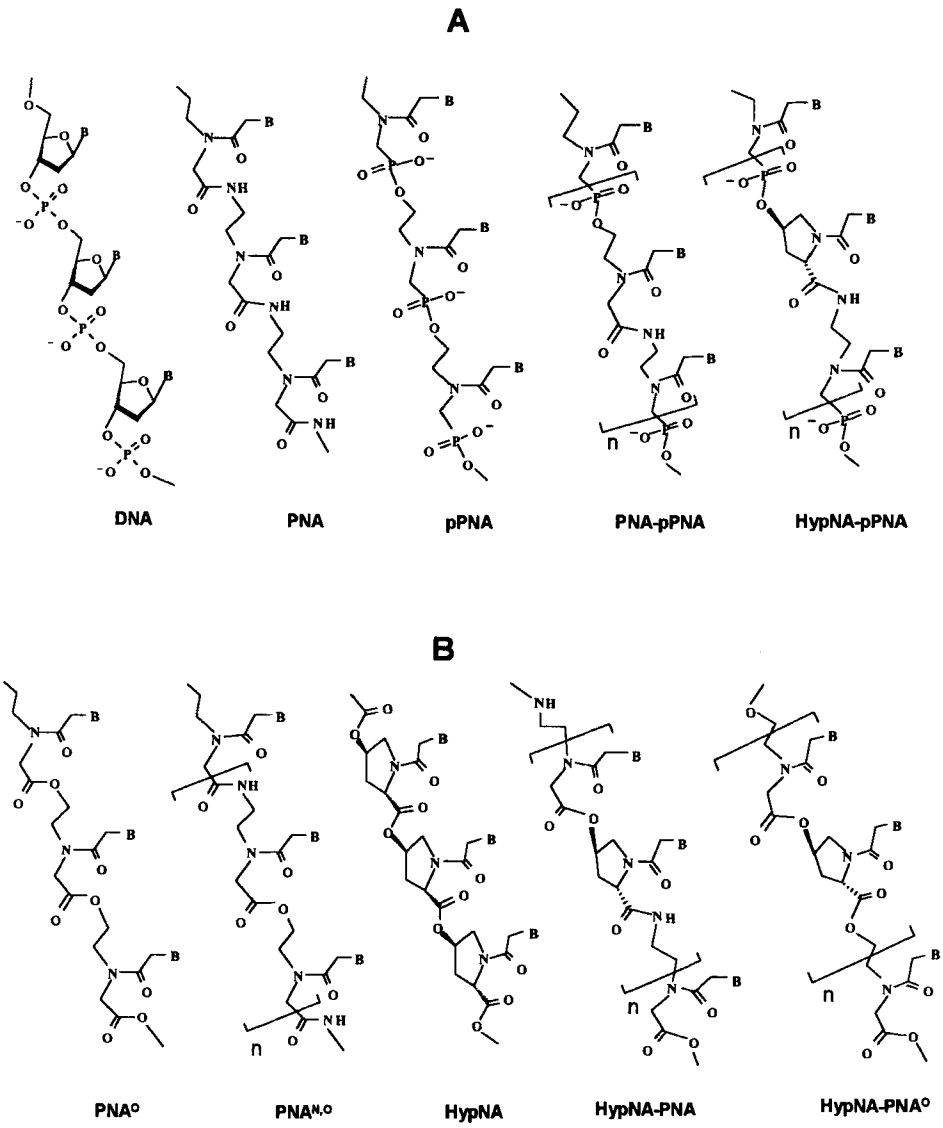


Figure 1. The structures of the PNA analogues and relative mimics.

pairing properties.[10,11] Similarly, we reported hetero-oligomer DNA mimics containing various amounts of pPNA monomers and PNA-like monomers on the base of *trans*-4-hydroxy-L-proline (HypNA) (Fig. 1).[12-14] This derivative of *trans*-4-hydroxy-L-proline represents conformationally constrained chiral PNA analogue, in which β-C atom of a hydroxyethyl unit and α-C atom of a glycyl unit of the backbone are bridged

by methylene group. The HypNA-pPNA hetero-oligomers demonstrated the stronger binding to complementary DNA and RNA strands in comparison with pure pPNAs and equivalent PNA-pPNA hybrids. Particularly, complexes formed by hetero-oligomers constructed of alternating pPNA and HypNA residues with complementary DNA (RNA) strands exhibited the stability very close to that of PNA/DNA(RNA) complexes.[12,14]

As is well known, the ester group models the amide group in its spatial characteristics, and it is quite similar to it in electron density distribution.[15] Mutual replaceability of amide and ester groups has been demonstrated on various biologically active peptides and depsipeptides.[16] One could therefore expect that analogues of PNAs, where in the backbone amide bonds are replaced by the ester linkages, would be very similar to the original compounds in their structure and binding properties. These premises were the starting point of our studies on the development of a set of novel PNA-related molecules and hybrids with variations in the backbone.

In this communication, we report PNA analogues, in which amide bonds are fully or partially substituted by ester linkages. These mimics were constructed of monomers on the base of N-(2-hydroxyethyl)glycine (PNA^{O}) as well as of *trans*-4-hydroxy-L-proline (HypNA). Moreover, the synthesis of modified PNAs derived from N-(2-methylaminoethyl)glycine (PNA^{Me}), which are also very close analogues of classical PNAs, is described (Fig. 1B). The effect of the introduction of these modifications into the PNA chain on the stability of mimic/DNA(RNA) complexes was examined.

Results and discussion

The structures of monomer units used in this study for the synthesis of PNA analogues are depicted in Fig. 2. The synthesis of PNA monomers with N-(2-aminoethyl)glycine (**1**) and N-(2-hydroxyethyl)glycine (**2**) backbone has been described by different authors.[17-20] We performed the preparation of these monomer building blocks with N-trityl N-or O-trityl- protecting groups using a three step simple general protocol developed by us earlier.[5] The synthesis of the N-methylated monomer **3** has been accomplished using a similar route. The starting compound was N- methyl--ethylenediamine. Its primary amino function was selectively trifluoroacetylated using ethyl trifluoroacetate in pyridine (a modification of the procedure published by O'Sullivan *et al.*[21]) with the following introduction of monomethoxytrityl (MMTr)

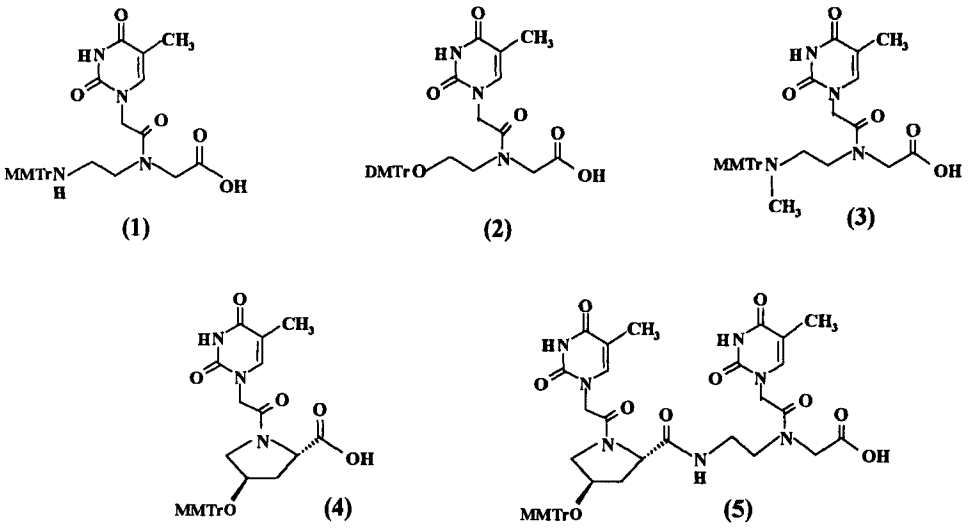
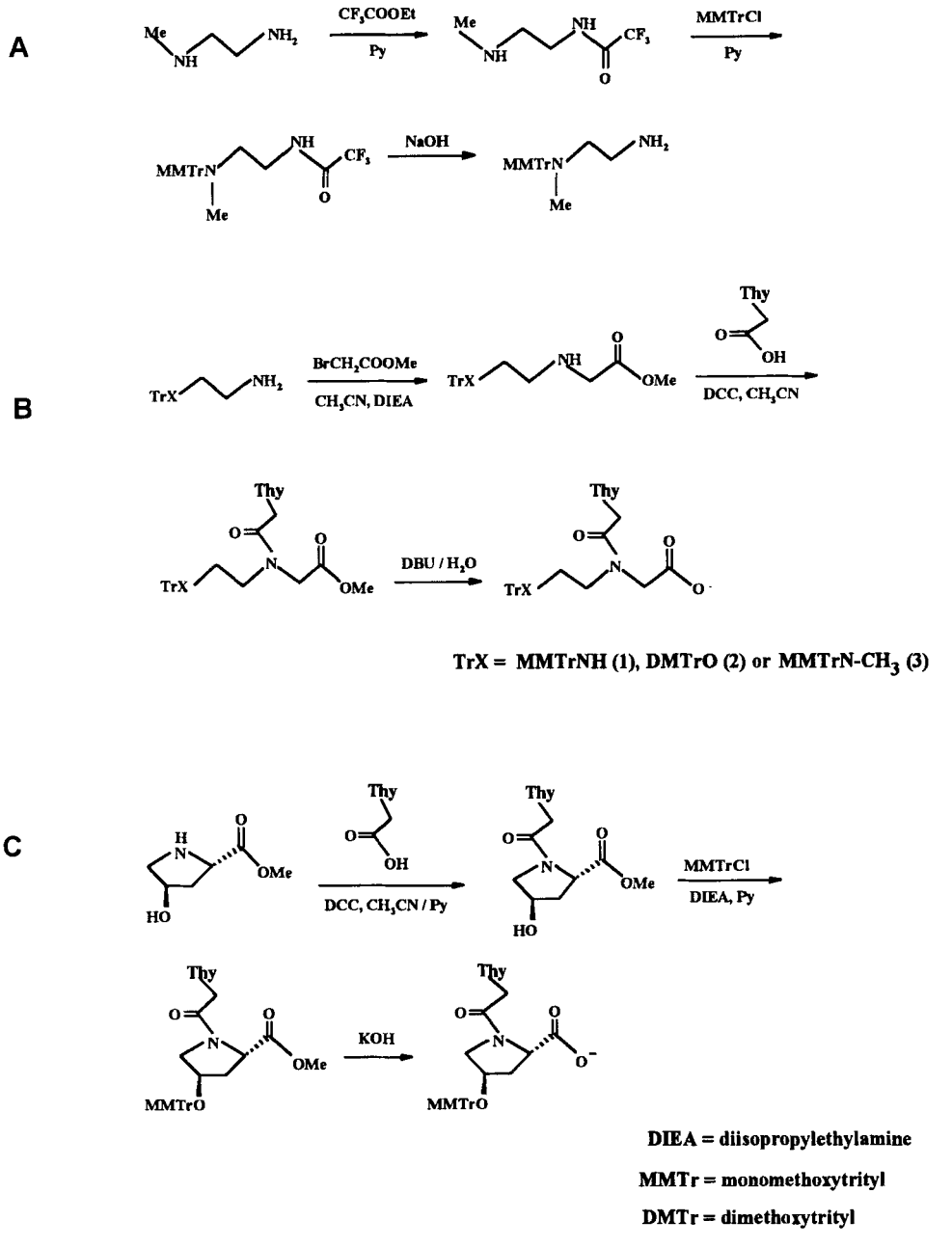


Figure 2. Chemical structures of building blocks for the synthesis of PNA-relative chimeric oligomers.

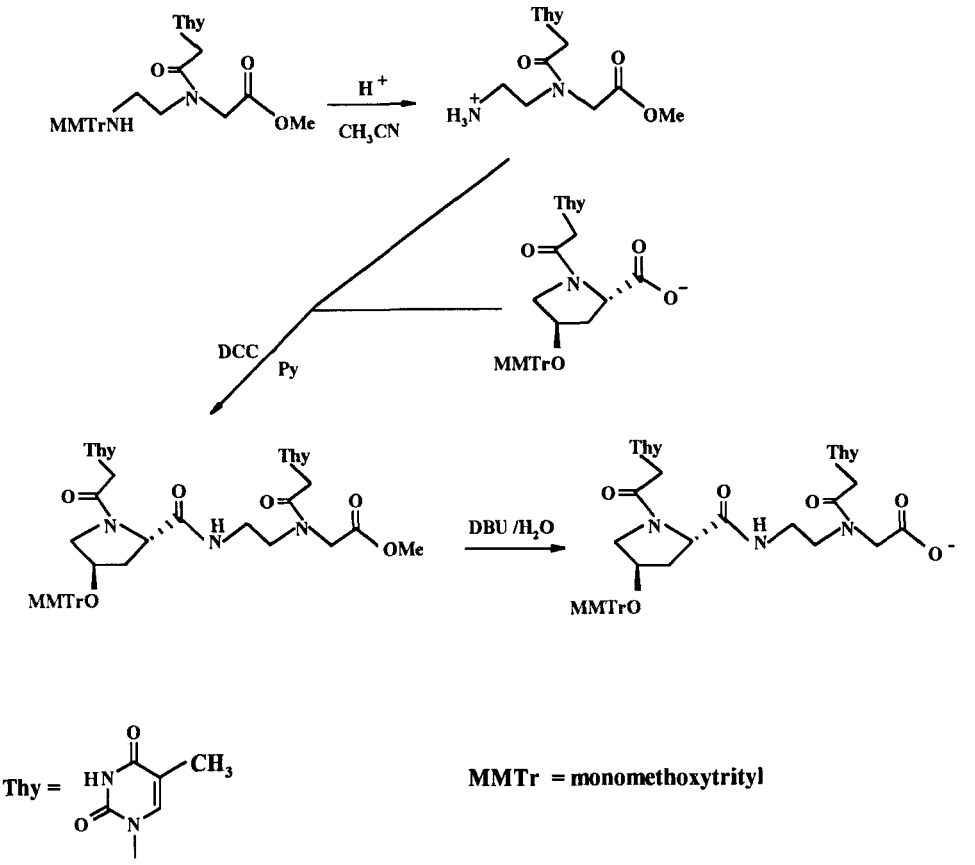
group to the secondary amino function (Scheme 1A). After the removal of trifluoroacetyl group, the resulting N-2-[(monomethoxytrityl)methylamino]ethylamine was transformed to the monomer **3** in three steps as depicted in Scheme 1B.

The synthesis of a monomer on the base of *trans*-4-hydroxy-L-proline (**4**) was achieved starting from the introduction of heterocyclic base by the coupling (thymin-1-yl)acetic acid with the secondary amino function of *trans*-4-hydroxy-L-proline methyl ester in the presence of a condensing agent in the conditions described for the synthesis of PNA and pPNA monomers previously [4,5] (Scheme 1C). The following monomethoxytritylation of a hydroxyl group gave fully protected monomer, which was converted into the desired compound **4** by the action of a base.[13] Very recently, an alternative scheme for the synthesis of the same type of a monomer unit has been published by J.H. van Boom and co-workers.[22]

The automated synthesis of chimeras has been accomplished starting from monomers **1** – **4** by the solid phase technique. The dimer building block **5**, which was obtained as depicted in Scheme 2, has been also used for the construction of mimic chains. The automated synthesis of oligomers was performed on an alkylsulfonylethyl-CPG support,[23] which was functionalized with p-chlorophenyl ester of 5'-dimethoxy-

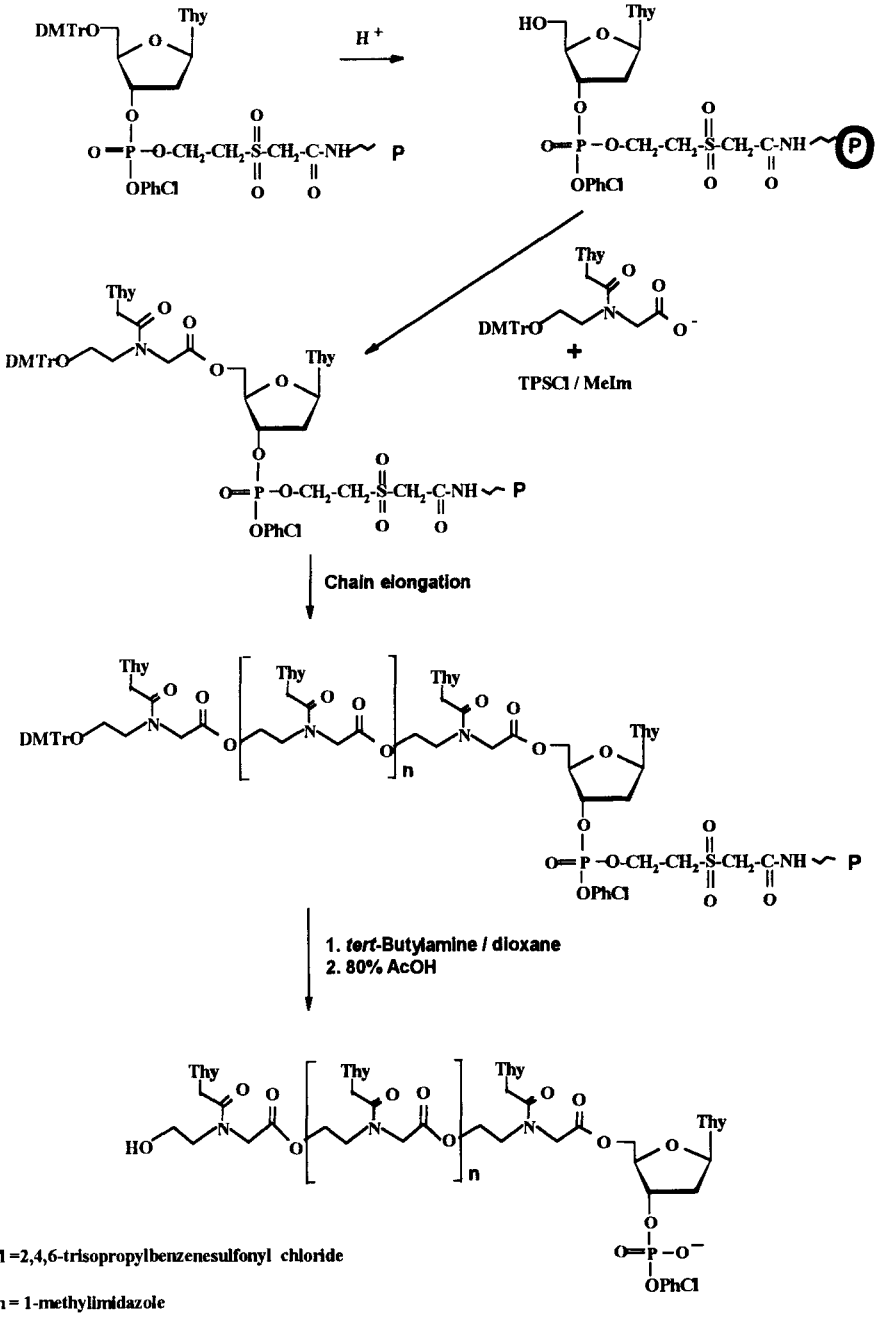


SCHEME 1



SCHEME 2

tritylthymidine 3'-phosphate as described [24] (Scheme 3). The synthesis and deprotection of regular PNAs was performed according to a protocol developed by us previously.[4-6] The synthesis of N-methylated PNAs was accomplished using monomer **3** and the same protocol. The synthetic cycle for the ester bond formation was in general similar to that for the synthesis of amide linkages (Table 1). For the formation of ester bond between carboxyl moiety of a monomer (or a dimer) unit and a terminal hydroxyl group on the support, 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in the presence of 1-methylimidazole (MeIm) was used as a condensing agent.[24] The condensations were performed in acetonitrile – pyridine mixture for 10-15 min. After the completion of chain elongation, oligomers containing ester bonds between monomers were cleaved



SCHEME 3

TABLE 1. Elongation cycles for the solid phase synthesis of oligomers[a]:

Step	Solvents and reagents	Time/min
Ester bond formation		
1. Detritylation	3% DCA in dichloromethane	1.5
2. Wash	Acetonitrile	0.5
3. Wash	Acetonitrile - pyridine (4:1, v/v)	1.0
4. Coupling	0.05 M COOH-component; 0.06 M TPSCl, 0.2 M 1-methylimidazole in acetonitrile - pyridine (2:1, v/v)	10 - 15
5. Wash	Acetonitrile - pyridine (4:1, v/v)	0.5
6. Capping	Ac_2O – MeIm - pyridine - acetonitrile (1:1:2:6, v/v/v/v)	1.0
7. Wash	Dichloromethane	1.0
Amide bond formation		
1. Detritylation	3% Pentafluorophenol in dichloromethane	3.0
2. Wash	0.2 M Diisopropylethylamine in dichloromethane	0.5
3. Wash	Acetonitrile - pyridine (4:1, v/v)	1.0
4. Coupling:	0.05 M COOH-component[b], 0.06 M TPSNT, 0.2 M 1-methylimidazole in acetonitrile - pyridine (2:1, v/v)	10.0
5. Wash	Acetonitrile - pyridine (4:1, v/v)	0.5
6. Capping	Ac_2O - MeIm - pyidine - acetonitrile (1:1:2:6, v/v/v/v)	1.0
7. Wash	Dichloromethane	1.0

[a] Reactions were performed using 30 mg of CPG support (1 μmol of the first nucleoside). [b] Before coupling, carboxylic component was pre-activated by mixing with 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole (TPSNT) and 1-methylimidazole.

from alkylsulfonylethyl-CPG support by the action of *tert*-butylamine. The latter reagent efficiently removed polyester oligomers from the support by the reaction of β-elimination for 1-2 h. On the other hand, it was mild enough to prevent cleavage of ester bonds between monomer units in the chain. After the removal of a terminal trityl group by the action of 80% acetic acid, polyester oligomer analogues were isolated by RP-FPLC (Fig. 3A) and characterized by mass-spectrometry. The structures and the yields of oligomers are shown in Fig 4A..

The examination of enzymatic stability of the mimics obtained revealed that they are fully stable to the action of exo- and endo-nucleases. The polyester HypNA oligomers

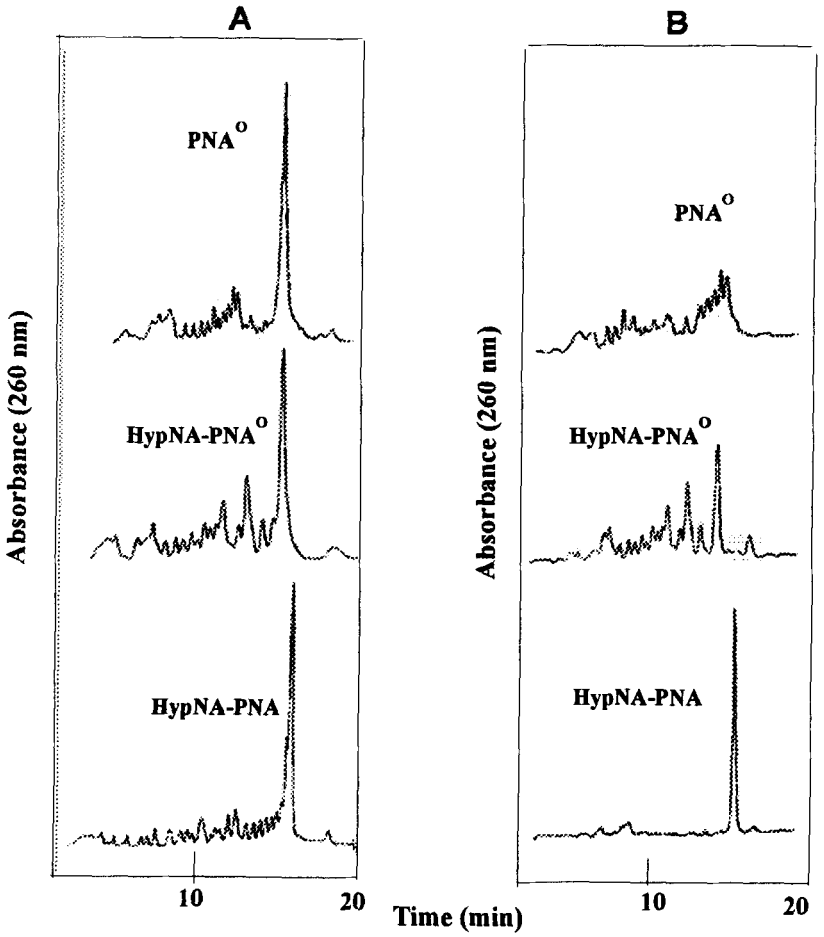


Figure 3. Reversed phase chromatography of polyester homo-Thy PNA analogues. **A**.- Isolation of crude oligomers after the deprotection. **B** - Analysis of the stability of purified oligomers after incubation at 40°C for 24 h in 0.05 M Tris-HCl (pH 8).

and HypNA-PNA hetero-oligomers were stable in water solutions. At the same time, we observed slow hydrolytic degradation of PNA^{O}, PNA^{N,O} and HypNA-PNA^{O} oligomers in water solutions at pH>7.5 (Fig. 3B), whereas they were stable at pH 6-7 for 3-5 days at room temperature.

Ultraviolet melting experiments on the hybridization properties of mimics revealed that PNA^{Me} oligomers are able to form rather stable complexes with complementary DNA and RNA strands. However, their stability was lower than that of complexes

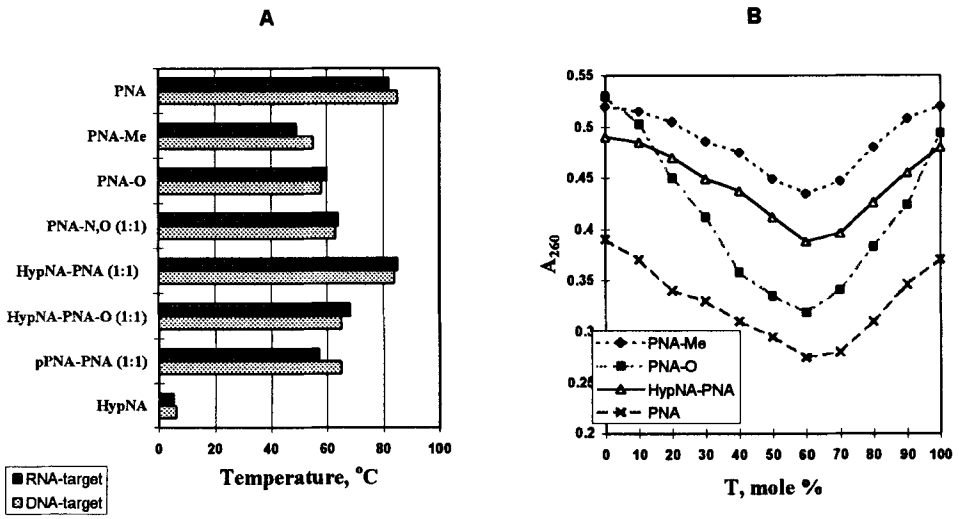


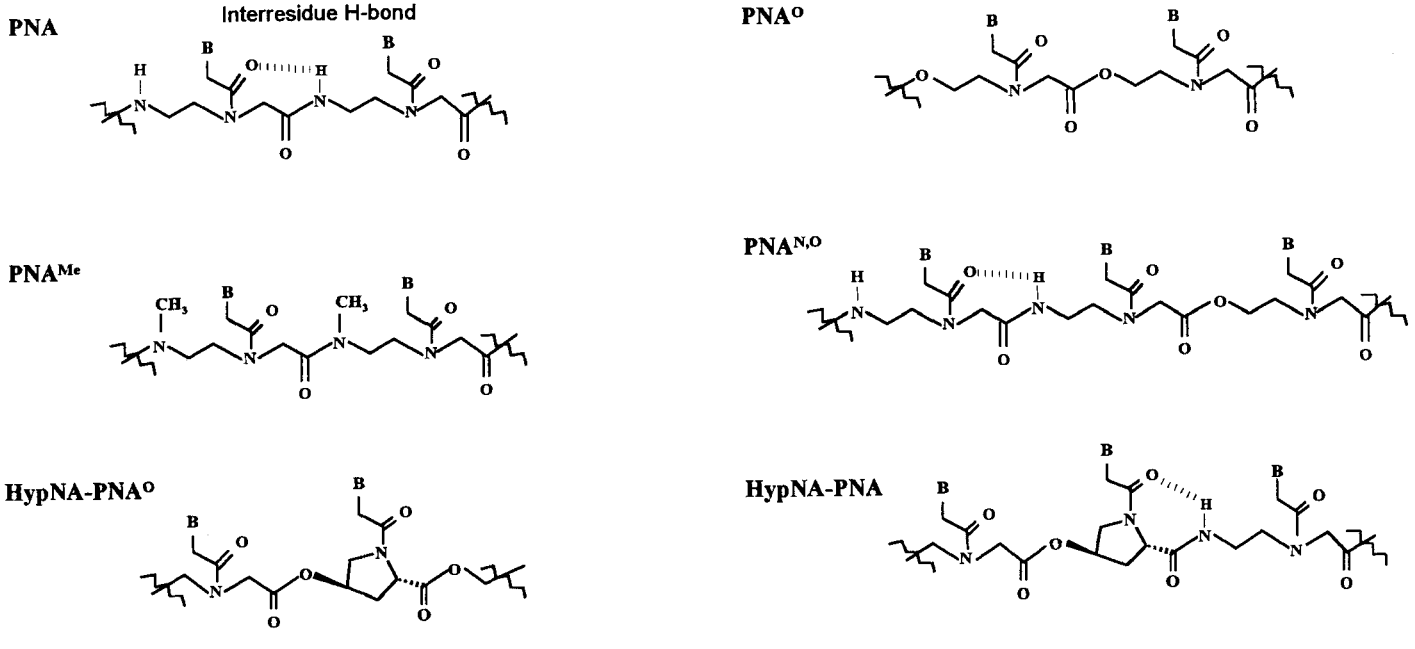
FIGURE 4. Hybridization properties of 16-mer homo-thymine mimics. (**A**) - Comparison of melting temperatures of complexes formed by 15-mer oligomers with complementary poly-A targets. (**B**) - Titration of dA_{15} target by complementary homo-Thy oligomers at 20°C in 0.1 M NaCl / 0.02 M sodium phosphate (pH 7) / 0.01 M $MgCl_2$ /0.1 mM EDTA.

formed by equivalent regular PNA oligomers. The stability of PNA^{O}/DNA(RNA) and PNA^{NO}/DNA(RNA) complexes was also lower than that of PNA/DNA(RNA) complexes (Fig. 4A) Their melting temperatures were close to those of the equivalent PNA-pPNA hetero-oligomers. Pure HypNA oligomer was not able to form stable complexes with DNA or RNA targets. In analogy to the published data on the improvement of base-pairing properties by the incorporation of *trans*-4-amino-L-proline derivatives into the PNA chain[10], the highest T_m values among the polyester analogues were found for hetero-oligomers with alternating PNA and HypNA residues. They show very strong binding to complementary DNA with equal, or even higher, T_m values than those exhibited by the equivalent regular pure PNA oligomers. From the titration data, it can be concluded that all above described homo-pyrimidine oligomers form triple helixes with deoxy- and ribo-homo-A templates (Fig. 4B).

Last time, much interest has been generated regarding the structure of complexes formed by PNA oligomers with complementary DNA or RNA targets.[25-27] Although detailed information on the structure of PNA strands in hetero-duplexes and triplexes

has been presented,[26,27] the high thermal stability of PNA/nucleic acid complexes has not been fully explained. One of the possible explanations is a reduction in electrostatic charge, which is associated with the replacement of a phosphodiester DNA backbone with a neutral PNA strand. This conclusion is in agreement with our data that the introduction of negative charges into the PNA chain in the case of pPNA analogues leads to a decrease in the stability of their complexes with DNA or RNA.[4-6] On the basis of molecular mechanics calculations and NMR data, it was proposed that the conformation of the PNA strand in its complex with the complementary nucleic acid strand is stabilized by inter-residue hydrogen bonding between the carbonyl oxygen nearest to the nucleobase and the backbone amide proton of the following PNA residue[25] (Scheme 4). In the case of polyester and N-methyl PNA analogues the formation of inter-residue hydrogen bonds is impossible. Nevertheless, PNA^{O} and PNA^{Me} oligomers are able to form quite stable complexes with complementary targets (Fig. 4A). These results argue against an essential role of the inter-residue hydrogen bond for the formation of PNA/DNA(RNA) complexes. From the other side, it was shown that DNA phosphate groups are hydrogen bonded to the PNA backbone amide protons of the Hoogsteen strand in PNA_2/DNA triple helices.[26] Most probably, the decreasing stability of $mimic_2$ /DNA(RNA) triplexes formed by modified oligomers containing ester or N-methylamide bonds between monomer units in comparison with classical PNAs may be attributed to their inherent inability to form such hydrogen bonds with phosphate groups of complementary nucleic acid chain.

From the present results, it can be concluded that amide groups of classical PNA backbone are not a prerequisite for the formation of stable complexes with complementary nucleic acid strands and can be substituted by ester or N-methylamide groups. Moreover, the incorporation of more rigid elements into the chain gives rise to the complex stability. Thus, the introduction of conformationally restricted HypNA units resulted in oligomer probes with improved hybridization properties. In view of the simplicity of the HypNA monomer synthesis, the hydrolytic and enzymatic stability of HypNA-PNA oligomers as well as their strong binding properties, this mimic type can be suggested as an alternative to PNA hetero-oligomers containing *trans*-4-amino-L-proline residues,[10] and, consequently, HypNA-PNA analogues can be considered as potential candidates for further evaluation as reagents for application in diagnostics and



SCHEME 4

therapy. Moreover, the above described PNA analogues will be useful for gaining a better understanding the contribution of the backbone to the recognition and stability of complexes formed by PNAs with nucleic acid strands.

Materials and methods

Solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker WM500 spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane. Mass spectra were recorded using either electrospray ionisation (ESI) or matrix-assisted laser desorption-ionization time of flight (MALDI-TOF). TLC was carried out on Merck Silica Gel 60 F_{254} plates in $CHCl_3$ - CH_3OH - triethylamine (8.4 : 1.5 : 0.1, v/v/v). Silica gel column chromatography was performed using Merk silica gel 60. Synthesis of oligomers was performed using Applied Biosystems Synthesizer 381A. Reversed phase separations were performed using Pharmacia ProRPC column/FPLC system and a linear gradient of acetonitrile (10 - 40%) in 0.1 M triethylammonium acetate (pH 7). The synthesis of PNA monomers (**1**) and (**2**) as well as synthesis of oligonucleotides, PNA oligomers and PNA-pPNA hybrids was accomplished as described previously.[4,5] Assays on the stability to the action of exo- (snake venom phosphodiesterase) and endonuclease (S_1 nuclease) were performed as described.[28] The hydrolytic stability assays of polyester oligomers were performed in Tris-HCl buffer at pH 6-9. Aliquots of the digestion products were analyzed by RP-FPLC.

N-{2-[N-(4-Methoxytrityl)-N-methyl]aminoethyl}-N-[(thymin-1-yl)acetyl]glycine (3). N-Methylethylenediamine (Aldrich) (0.93 ml,10 mmol) was dissolved in pyridine (25 ml), and ethyl trifluoroacetate (1.7 ml, 13 mmol) was added. After 2 h, the reaction mixture was evaporated in vacuum, the oil-like residue was dried by co-evaporation with pyridine and then dissolved in pyridine (30 ml) containing 1.5 ml of triethylamine. Monomethoxytrityl chloride (3.72 g, 12 mmol) was added. The mixture was allowed to stand for 2 h. After the treatment with 20 ml of 2 M NaOH in 50% methanol for 3 h, the mixture was extracted with benzene (2 x 70 ml). The organic fractions were evaporated to dryness, and the residue was purified by silica gel column chromatography in a methanole gradient (0-5%) in dichloromethane containing 0.5% triethylamine. The isolated N-2-[(monomethoxytrityl)-methylamino]ethylamine (8.6 mmol, 86%) was

dissolved in acetonitrile (40 ml) containing diisopropylethylamine (3.4 ml, 20 mmol). Methyl bromoacetic acid (0.95 ml, 10 mmol) was added, and after 20 min the reaction was terminated by the addition of water (80 ml). The resulted product was extracted with dichloromethane (2 x 50 ml). The organic fraction was washed with water (100 ml) and 5% $NaHCO_3$ (100 ml) and evaporated to dryness. The residue was co-evaporated with acetonitrile (2 x 20 ml) and dissolved in 40 ml of this solvent. Thymine-N^1-acetic acid (11 mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (12 mmol) were added, and the reaction mixture was shaken for 2 h. The reaction was terminated by the addition of water (5 ml) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (4 ml). After 5 h, the precipitate formed was removed by filtration, and the filtrate was evaporated to a gum. Compound (**3**) was isolated by silica gel column chromatography using a gradient of methanol (0-8 %) in CH_2Cl_2 / 1% triethylamine to yield 5.6 mmol (overall yield 56%) of the product. Rf 0.17; m/z = 571 $(M+H)^+[C_{32}H_{35}N_4O_6]$; ^{1}H-NMR ($CDCl_3$): 1.22 (t, 9H, $^+NHEt_3$-C*H*$_3$); 1.85 (s, 3H, Thy-*CH*$_3$); 2.15 (s, 3H, N-C*H*$_3$); 2.35 (m, 2H, MMTrN-C*H*$_2$) 2.98 (q, 6H, $^+NHEt_3$-C*H*$_2$); 3.65 (m, 2H, MMTrNH-CH$_2$-C*H*$_2$), 3.75 (s, 3H, MMTr-OC*H*$_3$); 3.95-4.05 (2xs, rotamers, 2H, C*H*$_2$-CO-O$^-$), 4.55-4.65 (2xs, rotamers, 2H, N-CO-C*H*$_2$); 6.75-7.45 (m, 15H, Ar-*H* and Thy-*H*-6); 8.4 (s, 1H, N*H*).

4-(4-Methoxytrityloxy)-*N*-[(thymin-1-yl)acetyl]-L-proline (4). Methyl ester of *trans*-4-hydroxy-L-proline hydrochloride (1.82 g, 10 mmol) (Bachem) was dissolved in 40 ml of pyridine - acetonitrile (1:1, v/v) containing triethylamine (1.4 ml, 10 mmol), and (thymin-1-yl)acetic acid (2.02 g, 11 mmol) and DCC (2.47 g, 12 mmol) were added under stirring. After 3 h, the reaction was terminated by the addition of water (2 ml), and the mixture was allowed to stand for 16 h at room temperature. The precipitate of N,N'-dicyclohexylurea was removed by filtration, the solution was evaporated to a gum, which was dried by evaporation with pyridine (2 x 30 ml) and dissolved in 40 ml of pyridine. Then, diisopropylethylamine (1.72 ml, 10 mmol) and 4-monomethoxytrityl chloride (4.01 g, 13 mmol) were added. The reaction mixture was heated at 70°C for 1 h and treated by 50 ml of 2M KOH in methanol - water (1:1, v/v) at 0°C. After 30 min, Dowex-50 (PyH^+) was added to achieve the neutral reaction. Dowex-50 was removed by filtration, washed with 60% aqueous pyridine (2 x 100 ml). Triethylamine (2.1 ml, 15 mmol) was added to a solution, which was concentrated under a reduced pressure. The residue was dried by evaporation with toluene, and the target compound was

isolated by silica-gel column chromatography in a gradient of methanol (0→8%) in dichloromethane, containing 1% of triethylamine. Yield 6.5 mmol (65%), R_f 0.28. m/z = 570 $(M+H)^+[C_{32}H_{31}N_3O_7]$; ^{1}H-NMR ($CDCl_3$): 1.20 (t, 9H, $^+NHEt_3$-C*H*$_3$); 1.8 (s, 3H, Thy-C*H*$_3$); 1.95-2.15 (m, 2H, 3'-*H*-Pro); 2.95 (q, 6H, $^+NHEt_3$-C*H*$_2$), 2.95-3.15 (m, 2H, 5'-*H*-Pro); 3.75 (s, 3H, MMTr-OC*H*$_3$); 4.0 (m, 1H, 4'-*H*-Pro); 4.25 (m, 1H, 2'-*H*-Pro); 4.4-4.5 (2xs, rotamers, 2H, N-CO-C*H*$_2$); 6.75-7.40 (m, 15H, Ar-*H* and Thy-*H*-6); 8.5 (s, 1H, N*H*).

HypNA-PNA dimer (5). Methyl ester of N-[2-(4-methoxytrityl)aminoethyl]-N-[(thymin-1-yl)acetyl]glycine (2 mmol) obtained as described earlier[5] was treated with picric acid (2.1 mmol) in acetonitrile - water (9.5 : 0.5 v/v, 10 ml) for 15 min. The reaction mixture was evaporated to dryness *in vacuo* and co-evaporated with acetonitrile (3 x 15 ml). The residue was dissolved in pyridine (8 ml) and a solution of compound (**4**) (2 mmol) in 6 ml of pyridine and DCC (3 mmol) were added. The reaction was completed in 1 h. Then water (1 ml) and DBU (7 mmol) were added to the reaction mixture. After 2 h, the precipitate formed was removed by filtration, and a solution obtained was evaporated to dryness and co-evaporated with toluene. The desired dimer (**4**) was isolated by silica gel column chromatography using a gradient of methanol (0 - 10%) in CH_2Cl_2 / 1.5% triethylamine with a 75% yield (1.5 mmol). R_f = 0.16; m/z = 1108 $(M+H)^+$ $[C_{63}H_{61}N_7O_{12}]$. ^{1}H-NMR ($CDCl_3$): 1.25 (t, 9H, $^+NHEt_3$-C*H*$_3$); 1.8 (s, 6H, 2 x Thy-C*H*$_3$); 1.95-2.15 (m, 2H, 3'-*H*-Pro); 2.9 (q, 6H, $^+NHEt_3$-C*H*$_2$); 2.95-3.15 (m, 2H, 5'-*H*-Pro); 3.3 (m, 2H, MMTrN-C*H*$_2$); 3.65 (m, 2H, MMTrNH-CH$_2$-C*H*$_2$), 3.75 (s, 3H, MMTr-OC*H*$_3$); 3.95-4.05 (2xs, rotamers, 2H, C*H*$_2$-CO-O$^-$), 4.2 (m, 1H, 4'-*H*-Pro); 4.45-4.65 (m, 5H, 2'-*H*-Pro and 2 x N-CO-C*H*$_2$); 6.75-7.45 (m, 16H, Ar-*H* and 2 x Thy-*H*-6).

Solid phase synthesis of oligomers. The CPG support was functionalized as described previously.[23,24] The synthesis and deprotection of PNA^{Me} oligo-mimetics were performed essentially as described by us previously for regular PNAs.[4,5] Chain elongation in the solid phase synthesis of PNA analogues containing ester linkages was carried out according to conditions given in Table 1 with the yields of 95-98% per step. Polyester oligo-mimics were cleaved from the support by the action of *tert*-butylamine - dioxane (1:1, v/v) for 1-2 h with the following removal of a terminal monomethoxytrityl protecting group by acidolysis (80% acetic acid, 15 min). In the case of oligomers

containing terminal amino function, the latter was capped by the action of acetic anhydride - 1-methylimidazole - pyridine (1:1:8, v/v/v) for 5 min after the removal of a monomethoxytrityl group and before the cleavage of oligomer from the support. Oligomers were purified by RP-FPLC, and their identity and purity were confirmed by mass spectrometry (MALDI-TOF).

Thermal denaturation studies. Melting curves of complexes formed by mimics with poly-A DNA(RNA) templates were measured at 260 nm from 5°C to 95°C using a Gilford 250 UV-VIS spectrophotometer equipped with a thermocontroller and a heating/cooling rate of 0.5°C/min. Solutions contained 3-5 μM of each oligomer in 100 mM NaCl/10 mM sodium phosphate (pH=7)/5 mM EDTA/10 mM $MgCl_2$. Melting temperature was taken to be the temperature of half-dissociation and was obtained from a plot of the derivative of $1/T$ vs absorbance. The following molar extinction coefficients were used: Ade 15.4 and Thy 8.8 $\mu mol^{-1} \cdot cm^{-1}$.

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