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# SYNTHESIS AND BINDING STUDY OF PHOSPHONATE ANALOGUES OF PNAs AND THEIR HYBRIDS WITH PNAs

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**ABSTRACT:** The preparation of monomers for the synthesis of phosphonate analogues of peptide nucleic acids containing the four natural nucleobases: thymine, cytosine, adenine and guanine, has been ccomplished. The monomers obtained were used for the automated online solid phase synthesis of pure phosphono-PNA oligomers as well as chimeras consisting of phosphono-PNA and PNA resudies. The hybridization properties of these oligonucleotide mimics to complementary DNA and RNA fragments were studied.

## INTRODUCTION

The application of oligonucleotides and analogues as antisense and antigene reagents has attached great attention due to their potential as therapeutics and diagnostic tools. Among a large number of modifications proposed to improve the oligonucleotides potency in terms of nuclease resistance, affinity to RNA and cellular uptake, peptide nucleic acids (PNAs) are currently of great interest due to their excellent binding properties and stability to nucleases[1]. However, the biggest obstacles to the use of PNAs as therapeutics are their poor water-solubility, tendency to self-aggregate, and low cellular uptake. In search of alternative compounds, which would overt these problems, a novel class of DNA mimics representing phosphonate PNA analogues (pPNAs) has been designed., and the synthesis of homo-pyrimidine pPNA oligomers of two types containing N-(2-hydroxyethyl)-phosphono glycine (pPNA-O), or N-(2-aminoethyl)-phosphono glycine (pPNA-N) backbone has been accomplished using solid phase[2,3] and solution[4] techniques.

## RESULTS AND DISCUSSION

To continue these investigations, we have accomplished the solid phase synthesis of three types of pPNA oligomers (pPNA-O, pPNA-N and pPNA-ON) containing all four nucleobases (Fig.1). A general synthetic route to obtain the pPNA thymine, adenine, guanine and cytosine monomers, which are

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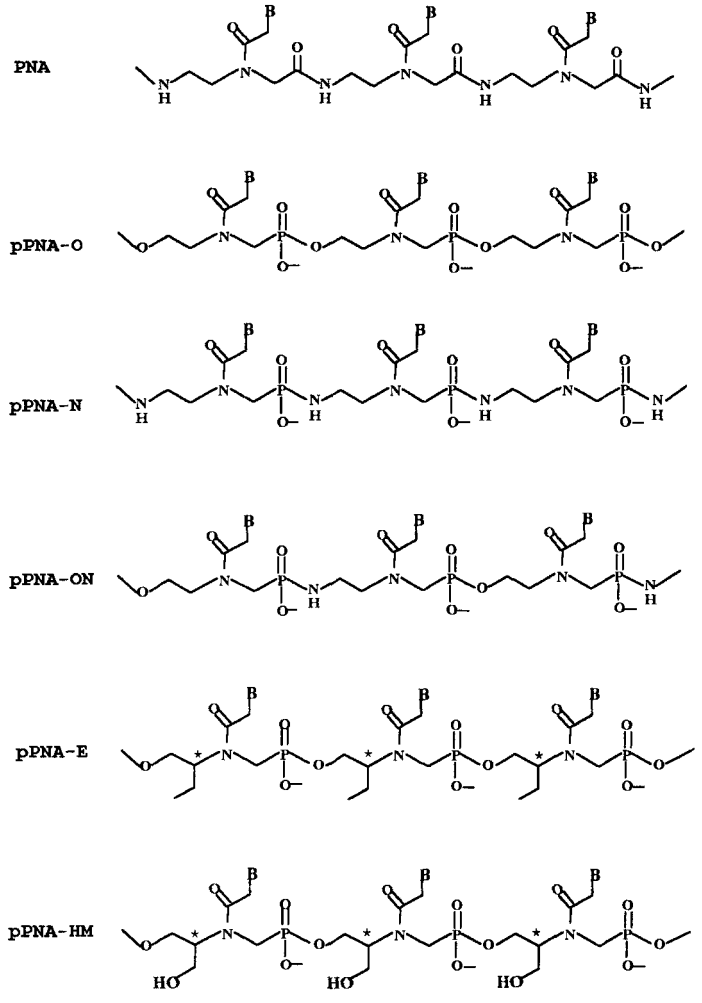


**Fig.1**. General structures of PNAs and phosphonate PNA analogues

compatible with the phosphotriester DNA synthesis with the use of intramolecular O-nucleophilic catalysis[5,6], has been developed on a preparative scale. The synthesis employs a common intermediate representing N-[2-(dimethoxytrityl)-hydroxyethyl]-aminomethyl, or N-[2-(monomethoxytrityl)-aminoethyl]-aminomethyl, diphenyl-phosphonate, to which any nucleobase can be attached via a methylene carbonyl linker (Fig. 2). As a variant, oligomers with a backbone consisted of alternated pPNA-O and pPNA-N residues were obtained by the solid phase synthesis starting from a specially constructed dimers (Fig.3g). Protocols for the solid phase synthesis of the DNA mimics obtained in this study are shown in Table 1.

The hybridization properties of pPNAs in comparison with corresponding DNA fragments were examined (Table 2). The results obtained indicate that all these types of pPNAs are able to form

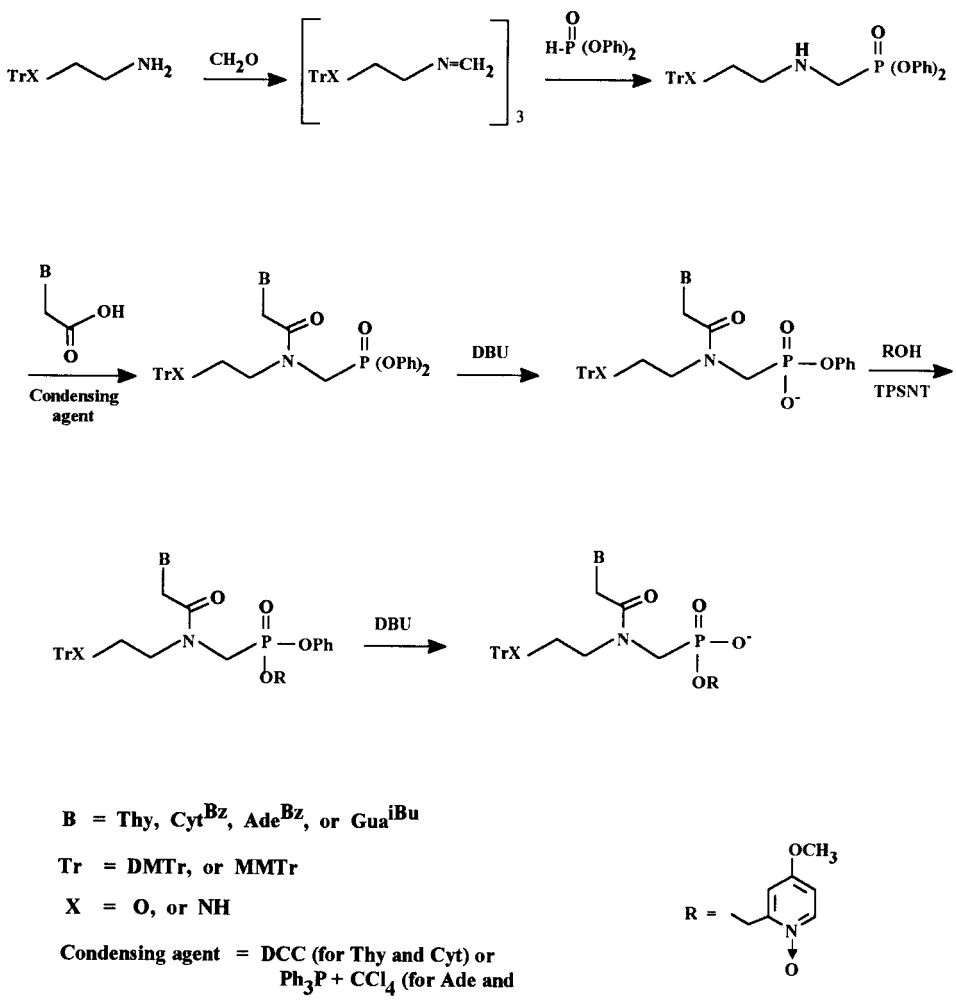


**Fig.2.** General scheme for the synthesis of a monomer for the construction of phosphono-PNAs by the solid phase phosphotriester method.

stable complexes with complementary DNA and RNA fragments as well as with complementary PNA and pPNA sequences (Fig. 4). The experiments on the titration of homo-pyrimidine and homo-adenine pPNAs with corresponding target oligonucleotides revealed that they form with the single-stranded complementary DNA, or RNA, triple helixes, whereas pPNAs containing three or all four nucleobases form duplexes.

We tried also to evaluate the effects of the orientation of thymine and cytosine consisting pPNA molecules as well as cytosine protonation on their binding to complementary nucleic acids sequences. The data obtained indicate that similar to PNAs [7], pPNAs have no preferential orientation to DNA and RNA targets. However, the T*m* values for complexes formed by oligomers in antiparallel orientation

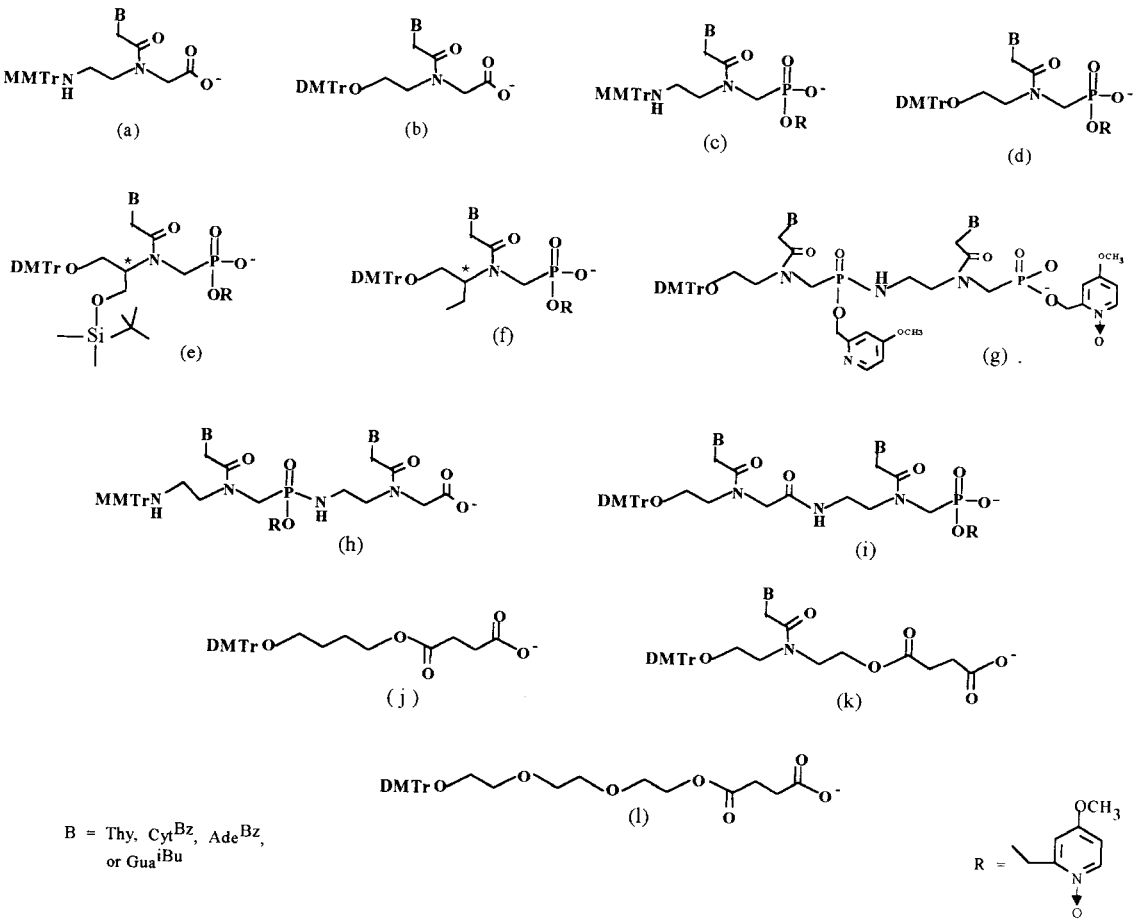


**Fig. 3.** Synthons for the automated phosphotriester synthesis of pPNAs and their hybrids with PNAs

**Table 1.** Protocols for the solid phase synthesis of chimeric oligomers*:

| Step | Solvents and reagents | Time (min) DNA and pPNAs | Time (min) PNAs |
|---|---|---|---|
| 1. Detritylation | 3% DCA in dichloromethane | 1.5 - 3.0 | - |
| | 3% Pentafluorophenol in dichloromethane | 3.0 | 3.0 |
| 2. Wash | Acetonitrile | 0.5 | - |
| | 0.25 M DIPEA in dichloroethane | - | 0.5 |
| 1. Wash | Acetonitrile - pyridine (4:1, v/v) | 3.0 | 3.0 |
| 2. Coupling | 0.05 M P-component; 0.15 M TPSNT in acetonitrile - pyridine (4:1, v/v) | 1.0 ( DNA) or 5-10 (pPNA) | - |
| | 0.05 M Monomer; 0.06 M TPSNT, 1-methylimidazole (0.15 M) in acetonitrile - pyridine (1:1, v/v) | - | 10.0** |
| 5. Wash | Acetonitrile | 0.5 | - |
| | Acetonitrile - pyridine (1:1, v/v) | - | 0.5 |
| 6. Wash | Acetonitrile | 2.0 | - |
| | Dichloroethane | - | 1.0 |

- Reactions were performed using 30 mg of a CPG support containing about 1 μmol of the trityl groups and Applied Biosystems DNA Synthesizer 381A.

** Before coupling, carboxylic component was pre-activated by mixing with TPSNT and 1-methylimidazole in one vessel.

were higher than those for complexes in parallel orientation that is similar to PNAs (Table 2). As it was expected, protonation of cytosines at pH 5.5 resulted in a small increase of the Tm values.

Moreover, a number of modified pPNA monomers including special synthons for functionalization of CPG supports has been prepared. Thus, optically active pPNA monomer analogue bearing thymine resudue was prepared from R(-)-2-amino-1-butanol by the route similar to that used for regular pPNA molecules (Fig. 3f). The second type of the modified pPNAs was derived from L-serine methyl ester and contained the additional OH-group on a side chain of the pPNA backbone (Fig. 3e).

These monomers were used in the automated synthesis of chiral oligo-Thy sequences, and their binding behaviour to natural poly-dA (or poly-rA) complement was assayed by UV spectroscopy and gel-electrophoresis. We have found that the oligomer obtained on the base of R(-)-2-amino-1-butanol forms triplexes with the targets, and its chirality arising from ethyl group incorporated as a side-chain of the backbone does not affect significantly the hybridization properties of the molecule. However, the Tm values for complexes formed by this oligomer are lower if compare to the Tm values of complexes formed by regular pPNAs (Table 2). At the same time, the introduction of a hydroxyl group into the

**Table 2.** Melting temperatures of complexes formed by pPNAs and PNA-pPNA hybrids with complementary oligonucleotide targets.

| Compound | Sequence | Tm (°C) [b] | |
|---|---|---|---|
| | | d- template | r- template |
| 1 | (T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*)-dT | 49 | 28 |
| 2 | (T*-T"-T*-T"-T*-T"-T*-T"-T*-T"-T*-T"-T*-T")-dT | 63 | 65 |
| 3 | (T*-T*-T*-T*-T*-T*-T*-T*)-d(T-T-T-T-T-T-T) | 20 | 35 |
| 4 | (T*-T*-T*-T*-T*-T*-T*)-(t*)-(t-t-t-t-t-t)-d(NHT) | 53 | 57 |
| 5 | Ac-(t-t-t-t-t-t-t)-(T")-(T*T*T*T*T*T*T*T*T*T*)-dT | 52 | 58 |
| 6 | (t*-T"-t*-T"-t*-T"-t*-T"-t*-T'-t*-T"-t*-T")-dT | 67 | 58 |
| 7 | (T"-t-T"-t-T"-t-T"-t-T"-t-T"-t-T"-t)-d(NHT) | 55 | 52 |
| 8 | Ac(t-t-t-t-t-t-t-t-t-t-t-t-t-t-d(NHT) | 85 | 80 |
| 9 | (T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*-T*)-dT (chiral) | 38 | 30 |
| 10 | (A*-A*-A*-A*-A*-A*-A*-A*-A*-A*-A*-A*-A*-A*)-dA | 46 | 45 |
| 11 | (C*-T*-T*-T*-C*-T*-T*-T*-T*-C*-T*-C*-T*)-dT (anti-par.) | 50 | 45 |
| 12 | (T*-T*-C*-T*-C*-T*-T*-T*-T*-C*-T*-T*-T*)-dC (par.) | 43 | 32 |
| 13 | (T*-C*-A*-C*-T*-C*-A*-A*-C*-A*-C*-T*-C*-A*)-dC (anti-par.) | 37 | 40 |
| 14 | (C*-T*-G*-C*-A*-A*-A*-G*-G*-A*-C*-A*-C*-C*-A*-T*-G*)-dA(anti-par) | 45 | 42 |
| 15 | (C"-t*-G"-c*-A"-a*-A"-g*-G"-a*-C"-a*-C"-c*-A"-t*-G")-dA(anti-par) | 59 | 61 |
| dT15 | d(T-T-T-T-T-T-T-T-T-T-T-T-T-T-T) | 43 | 45 |

**a** - (t) - PNA residue (1); (t*, c*, a*, g*) - PNA residue of type ( 2 ); (T*, C*, A*, G*) - pPNA residue of type (4 ); (T") - pPNA residue of type (3 ) ; d(NHT) - residue of 5'- aminothymidine

**b** - In the cases, when dissociation curves had two and more transitions, the Tm value corresponding to the highest transition is given.

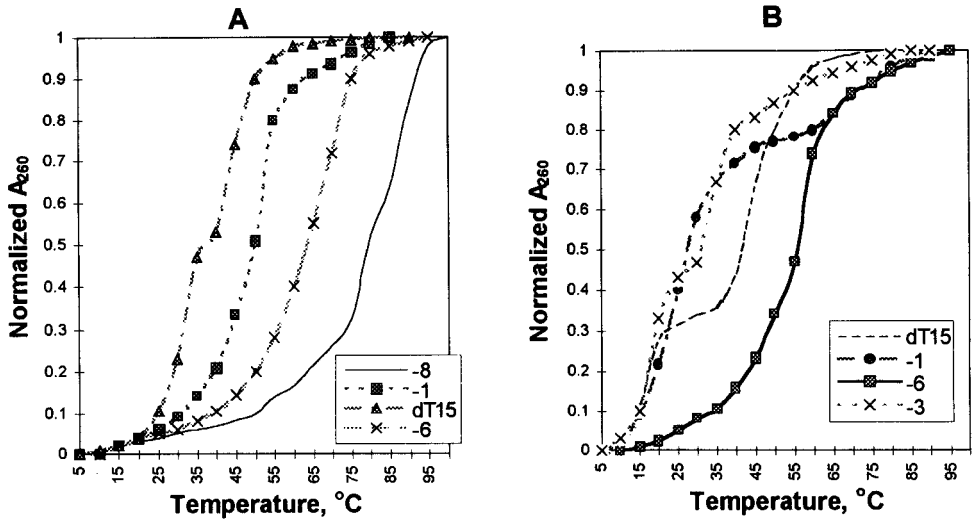


**Fig.4.** Melting curves of complexes formed by DNA mimics (1, 3, 6 and 8) or $dT_{15}$ oligomers with $dA_{15}$ (A) or $rA_{15}$ (B) targets. Solutions containing 3-5 μM of each oligomer in 150 mM NaCl / 10 mM Tris-HCl (pH 7-8) / 5 mM EDTA /10 mM $MgCl_2$ were heated at 95°C for 2 min and then allowed to cool slowly to 5°C before data collection. The changes in absorbance at 260 nm versus temperature were measured with a heating rate 0.5°C/min from 5-95°C.

side chain of the backbone had dramatically destabilizing effect. The oligomer synthesized from a monomer derived from L-serine was not able to form stable complex with complementary target. A steady increase in absorbance was observed during the melting experiments with a mixture of this oligomer with complementary deoxy and ribo targets, but no cooperative transitions were detected. We did not also detect complex formed by hydroxyl containing oligomer with poly-A target by gel-electrophoresis.

It should be noted that the introduction of negative charges into the PNA backbone lead to the excellent solubility characteristics of pPNAs. These molecules are stable to the action of nucleases, and they have good binding properties to complementary DNA and RNA fragments, however the T*m* values of such complexes are lower when compared to those of corresponding PNAs sequences. In order to improve physico-chemical and biological properties of both PNAs and pPNAs, the automated synthesis of chimeric oligomers consisted of alternated PNA and phosphono-PNA residues has been accomplished (Fig. 5, PNA-pPNA-1 and -2). PNA-pPNA chimeric oligomers were synthesized in a stepwise fashion using pPNA monomers in conjunction with standard PNA monomers containing the monomethoxytrityl protected primary amino function [8,9] or with analogues of PNA monomers containing the N-(2-hydroxyethyl)glycine backbone unit with dimethoxytrityl group for temporary protection of the hydroxyl

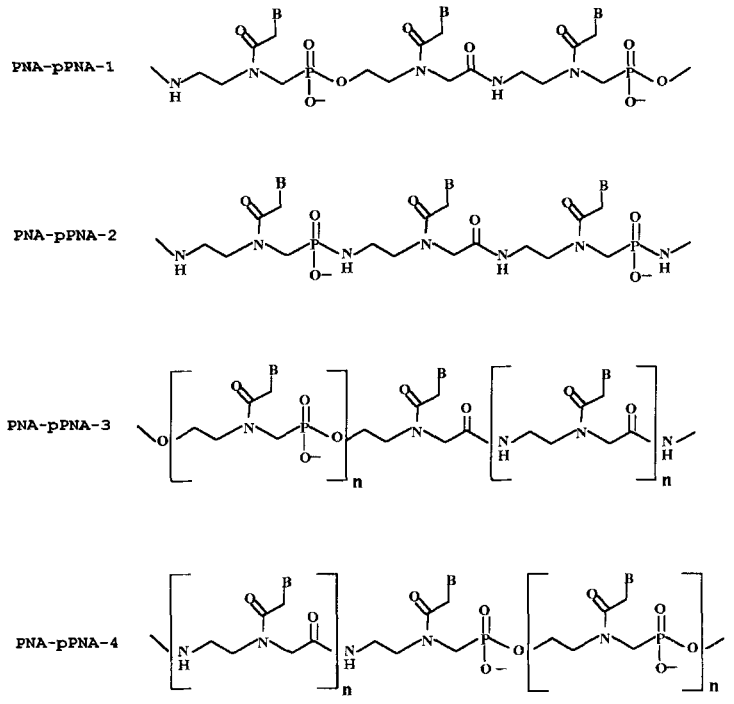


**Fig. 5.** General structures of PNA-pPNA hybrids synthesized.

function[10] (Fig. 3a,b). Specially constructed PNA-pPNA and pPNA-PNA dimers were also used for the synthesis of chimeras composed of alternating PNA and pPNA monomers (Fig. 3 h, i). The second type of PNA-pPNA hybrids obtained was constructed from PNA and pPNA stretches (Fig. 5, PNA-pPNA-3 and -4). The hybridization properties of PNA-pPNA chimeric oligomers to DNA and RNA complementary strands in comparison with pure PNAs, pPNAs, DNA-pPNA hybrids and DNA fragments as well as the nuclease stability of these compounds were examined. UV melting experiments and non-denaturing gel retardation assays confirmed that these chimeras form stable complexes with complementary DNA and RNA fragments. They formed more stable complexes with nucleic acids than do the equivalent DNA and DNA-pPNA hybrids. Some increase in stability of complexes formed by PNA-pPNA hybrids with DNA and RNA targets with respect to the similar complexes formed by pure pPNAs was also observed. The molecules with alternated PNA and pPNA residues gave more stable complexes than the hybrids constructed from PNA and pPNA stretches. We have found that homo-pyrimidine PNA-pPNA chimeras form with the single-stranded complementary DNA or RNA template triple helixes without detectable double-helical intermediate, where as chimeras with mixed purine/pyrimidine sequences gave duplexes. These chimeras as well as pure pPNAs and PNAs were found to be highly resistant to degradation by VPDE and nuclease $S_1$.

## CONCLUSION

We have demonstrated that pPNAs of various types and PNA-pPNA chimeras can be automatically synthesized using synthons, which are compatible with the DNA phosphotriester synthesis. It was confirmed that these nucleic acids mimics hybridize specifically to complementary DNA and RNA single-stranded fragments as well as to complementary PNA sequences and themselves. Among the compounds obtained in this study, the PNA-pPNA chimeras constructed from these isosteric types of molecules are very promising for further evaluation as potential therapeutic agents.

## REFERENCES

1. P.E.Nielsen, M.Egholm, R.H. Berg, O.Buchardt *Science*, **1991**, ***254***, 1497.
2. A.C.van der Laan, R.Stromberg, J.H.van Boom, E.Kuyl-Yeheskiely, V.A.Efimov, O.G.Chakhmakhcheva *Tetrahedron Letters*, **1996**, ***37***, 7857.
3. V.A.Efimov, M.V.Choob, A.L.Kalinkina, O.G.Chakhmakhcheva, R.Stromberg, A.C. van der Laan, N.J.Meeuwenoord, E.Kuyl-Yeheskiely, J.H.van Boom *Collect. Czech. Chem. Commun.*, **1996**, ***61***, s262
4. A. Peyman, E. Uhlmann, K. Wagner, S. Augustin, G. Breipohl, D.W.Will, A.Schafer, H.Wallmeier *Angew. Chem. Int. Ed. Engl.*, **1996**, ***35***, 2636.
5. V. Efimov, A Buryakova, I.Dubey, N.Polushin, O.Chakhmakhcheva, Y.Ovchinnikov *Nucleic Acids Res.*, **1986**, ***14***, 6526.
6. T.Szabo, A.Kers, J.Stawinski *Nucleic Acids Res.*, **1995**, ***23***, 893.
7. E.A. Lesnik,, L.M. Risen, D.A. Driver, M.C. Griffith, K. Sprankle, S. Frier. *Nucleic Acids Res.*, **1997**, ***25***, 568.
8. A.C. van der Laan, N.J. Meeuwenoord, E. Kuyl-Yeheskiely, R.S. Oosting,, R. Brands, J.H.van Boom *Rec. Trav. Chim, Pays-Bas.*, **1995**, ***114***, 295.
9. E. Uhlmann, D.W.Will, G.Breipohl, D.Langner, A. Ryte *Angew. Chem. Int. Ed. Engl.*, **1996**, ***35***, 2632.
10. K.H.Petersen, D.K.Jensen, M.Egholm, P.E.Nielsen, O.Buchardt *Bioorganic & Medicinal Chemistry Letters*, **1995**, ***5***, 1119.