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New Approach to Solid Phase Synthesis of Polyamide Nucleic Acids Analogues (PNA) and PNA-DNA Conjugates

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Abstract:

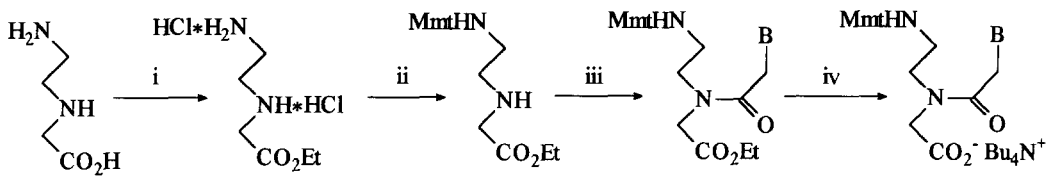
New version of sequential DNA and polyamide solid phase synthesis is applied to the preparation of PNA and PNA-DNA conjugates bearing free 3'-end. N-Mmt protected uracil and thymine PNA monomers are obtained in high yields. Novel support derived from TSK Gel Toyopearl® and efficient coupling procedure with 2,4,6-triisopropylbenzenesulfonyloxy-1-benzotriazole (TPSOBt) are also developed. Preliminary results of biological testing of prepared compounds are reported.

For the last few years since discovery PNAs[1,2] attracted an increasing attention in order to use their remarkable affinity to complementary DNA (RNA) sequences.[3-5] However a high structural difference between natural nucleic acids and PNA[2] precludes their enzymatic recognition. If the hypothetical mixed oligomers combined the PNA sequence-specific binding ability and nuclease resistance with DNA properties such as priming capacity their application could embrace DNA sequencing and PCR. This paper presents a new solid phase procedure for synthesis of PNAs and PNA-DNA conjugates bearing 3'-end necessary for DNA polymerase extension in addition with sites for non-isotopic labelling. The compounds incorporate uracil which facile electrophilic substitution at 5-carbon is well established.

PNA assembling methods available[6] hardly permit the sequential solid phase oligodeoxynucleotide and polyamide synthesis due to drastic acidic conditions that inevitably would damage sensitive nucleosides. Therefore we chose weak acid labile 4-methoxytrityl group (Mmt)[7] as temporary amine protection. The conditions generally used for Mmt removal (5% w/v TCA in DCE, 2-4 min) are known to cause little depurination in DNA synthesis. In our hands Mmt protection completely resisted capping conditions (Ac_2O/ DMAP/2,6-lutidine/THF, RT, 6 hrs) while uracil was noticeably acetylated within few minutes. An advantage of the Mmt group is possible direct monitoring of coupling efficiency by measuring absorbance of carbenium ion released in acid (ε_{478} = 65000). Conventional ninhydrin test is here impracticable due to small scale of routine DNA synthesis. Besides, the Mmt group increases much the solubility of protected monomers and may be used as purification handle on RP-HPLC column.

An uracil residue usually doesn't need any protection though some modification may occur during the synthesis.[8] Therefore we investigated a new coupling procedure for the uracil PNA synthesis using various arenesulfonates of 1-hydroxybenzotriazole.[9] 2,4,6-Triisopropylbenzenesulfonyl derivative (TPSOBt) proved to be the most promising condensing reagent that caused no detectable modification of heterocycle with coupling efficiency compared to commercially available PyBOP®.[10] TPSOBt may be obtained as a crystalline nonhygroscopic compound (mp.81-83°C from aq. iPrOH) and stored at RT without decomposition for at least 3 years. Our experiments showed the reagent didn't significantly react with polymer-supported unprotected primary amine during condensation on the contrary to more reactive coupling reagents.[11]

In order to develop the polymer support that would fit both polyamide and oligonucleotide synthesis we chose TSK Gel Toyopearl® recently employed in DNA synthesis.[12] Good properties of the resin together with insertion of polyethylene glycol (PEG) residues allowed us to obtain the analogue of PEG-polystyrene or TentaGel®[13] suitable for peptide and large scale oligonucleotide synthesis. The first PNA monomer unit was attached to the support via alkali labile ester linkage (TPS-Cl/NMI procedure, loading 207 μmol/g).



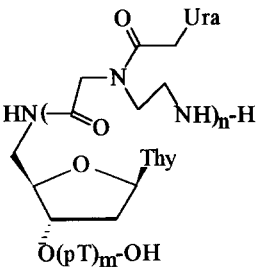
1 **2** **3** **5a** B = Ura **6a** B = Ura

5b B = Thy **6b** B = Thy

i.7M HCl/EtOH, reflux, 2 h (72%). ii. Mmt-Cl/NEt_3/DCM, 0°C, 1 h (99%). iii. **4a-b**/NEt_3/MeCN, RT, 18 h (**5a** 71%, **5b** 80%). iv. 0.2M NaOH aq./dioxane, RT, 2-4 h, n-Bu_4N^+ HSO_4^- (**6a** 88%, **6b** 87%).

Uracil PNA monomer was synthesized as follows. N-2-aminoethylglycine[14] **1** (mp.151-153°C; lit.[14] mp.152-153 °C) was converted to its ethyl ester dihydrochloride **2** (mp.172-174°C) and selectively monoprotected by Mmt-Cl giving almost pure oily **3** in excellent yield. Uracil-1-yl acetic acid (mp.294-296°C) was prepared as described for thymin-1-yl acetic acid[15] and treated with DCC and PfpOH in DMF to give pentafluorophenyl ester **4a** (88%, mp.219-221°C). The main building block **5a** was obtained by reaction of **4a** with **3** as white foam after short column purification. Subsequent saponification and conversion to tetra-n-butylammonium salt led up to desired uracil PNA monomer **6a** as white powder after precipitation into n-hexane. Thymine PNA monomer **6b** (R=Me) was synthesized by the same procedure.[16] Noteworthy **6b** is more soluble (up to 0.5M in MeCN) than commercially available Boc-protected thymine acid.

The solid phase assembling of model uracil PNA decamer was accomplished following standard peptide and oligonucleotide synthesis protocols.[17] The average stepwise yield was 96% (as estimated by Mmt^+ absorbance). After nine elongation cycles crude oligomer was detached from polymer by alkaline hydrolysis



PNA-DNA conjugates	T_m,°C[21]
7a n = 5, m = 4	-
7b n = 10, m = 4	65.0
7c n = 10, m = 6	67.5
7d n = 10, m = 9	70.0

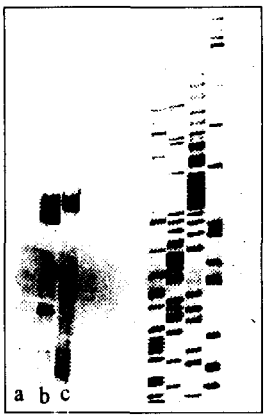


Fig. 1. Termination of DNA synthesis *in vitro*.[22] Line a - no termination, line b - PNA decamer, line c - conjugate **7b**.

and purified on RP-HPLC column using the retained Mmt group as a lipophilic handle. PNA decamer resulted was rechromatographed after deprotection (80% aq.AcOH, RT, 1 h).[18]

Several PNA-DNA conjugates **7a-d** were synthesized in two step procedure.[19] The oligothymidine part was automatically assembled by the standard cyanoethyl phosphoramidite method using modified 5'-deoxy-5'-Mmt-aminothymidine phosphoramidite[20] in the last cycle. Uracil PNA synthesis then occurred manually as described for PNA .[17] Stepwise yields were somewhat lower and varied from 91 to 99%. After synthesis was completed the oligomers were subjected to conventional ammonolytic cleavage (RT, 1 h) and the conjugates were then purified by RP-HPLC.[19]

Displacement of thymine by uracil in PNA results in a slight decrease of T_m. Hybridization of the uracil PNA decamer[21] revealed T_m value of 65°C instead of 73°C for thymine analogue.[2] Conjugates **7c-d** demonstrated higher T_ms apparently due to 3'-DNA part contribution. According to our experiments both uracil PNA decamer and PNA-DNA conjugate **7b** specifically terminated DNA synthesis *in vitro* promoted by Klenow fragment at 37°C on ss template (fig.1)[22] with the position of observed termination corresponding to the expected site of PNA binding. 3'-Oligodeoxynucleotide part of conjugate **7b** was also demonstrated to be recognized by 3'-deoxynucleotidyl transferase with α-[^{32}P]-dATP attachment efficiency almost the same as decathymidylate while decamer PNA didn't display such a property.

Our results thus confirm uracil PNA and PNA-DNA conjugates described here[23] possess a high binding ability of normal PNAs and may be the substrates in enzymatic reactions concerned with DNA. Further attempts to investigate these conjugates as PCR primers and probes in direct DNA mapping by the scanning electron microscopy are now in progress.

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1. Other abbreviations used: Ac - acetyl, Boc - tert-butoxycarbonyl, DCC - dicyclohexylcarbodiimide, DCE - 1,2-dichloroethane, DCM - dichloromethane, DIEA - diisopropylethylamine, DMAP - 4-dimethylamino-pyridine, DMF - N,N-dimethylformamide, HOBt - 1-hydroxybenzotriazole, NMI - 1-methylimidazole, PyBOP® - benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, TCA- trichloroacetic acid, THF - tetrahydrofuran.
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16. All compounds exhibited correct ^{1}H-NMR data. **6a** ($CDCl_3$, δ, ppm): 7.42-7.18 (m, 12H, Ar*H*); 7.14-7.12 (d, H-6), $J_{6,5}$=7.3 Hz; 6.77 (d, 2H, Ar*H*); 5.61-5.59 (d, H-5); 4.61 (s, 2H, CH_2CON); 3.87 (s, 2H, $COCH_2$); 3.74 (s, 3H, OCH_3); 3.54, 2.30 (bm, 4H, CH_2CH_2); 3.28 (m, 8H, $^+NCH_2$); 1.63, 1.40 (2m, 16H, CH_2); 0.98 (m, 12H, CH_3).
17. General protocol of uracil PNA synthesis (1 μmol column of DNA synthesizer MilliGen/Biosearch Model 7500, 15 mg of monomer-functionalized PEG-TSK Gel support): (1) DCE wash, 0.5 ml, 1 min. (2) Mmt deprotection, 5% w/v TCA/DCE, 5×0.5 ml, 5×1 min. (3) DCE wash, 2×0.5 ml, 2×1 min. (4) MeCN wash, 2×0.5 ml, 2×0.5 min. (5) Condensation: **6a** (5 eq, 0.1M), TPSOBt (6 eq), HOBt (6 eq) and DIEA (10 eq) in MeCN, 150 μl, 50 min. (6) MeCN wash, 0.5 ml, 0.5 min. (7) THF wash, 0.5 ml, 0.5 min. (8) Capping (omitted at the last cycle): 0.25M Ac_2O and 0.05M DMAP in 2,6-lutidine - THF 1:9 v/v, 200 μl, 10 min. (9) THF wash, 2×0.5 ml, 2×0.5 min. (10) DCE wash, 2×0.5 ml, 2×0.5 min.
18. Crude oligomers were purified on C_{16} column using linear gradient of 80% aq.MeCN (buffer B) in 0.1M NEt_3×AcOH with 5% MeCN (buffer A). Satisfactory ES-MS analysis of purified PNA was obtained.
19. Oligonucleotide synthesis was performed on ABI 381B DNA synthesizer on T-derivatized LCAA-CPG in 1 μmol scale. Reaction column was then replaced from machine and subjected to manual PNA synthesis as described. Unexpected loss of N-terminal Mmt group was observed after ammonolysis.
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21. T_m was measured in 10 mM Tris×HCl buffer, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl on dA_{20} template with equimolar concentrations of counterparts.
22. DNA synthesis was primed by radioactive labelled M13 universal primer on M13 mp18 phage recombinant DNA with dA_{14} insertion. Conjugate **7a** didn't stop DNA synthesis at 37°C.
23. When present work had been almost finished another article related to the same subject appeared: Bergmann, F., Bannwarth, W., Tam, S. *Tetrahedron Lett.* **1995**, *36*, 6823-6826.

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