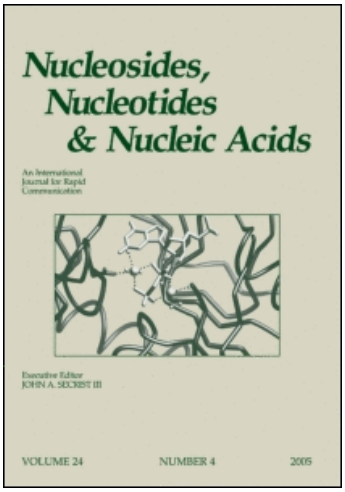


**Nucleosides, Nucleotides and Nucleic Acids**
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**To cite this Article** Lutz, M. J. , Will, D. W. , Breipohl, G. , Benner, S. A. and Uhlmann, E.(1999) 'Synthesis of a Monocharged Peptide Nucleic Acid (PNA) Analog and Its Recognition as Substrate by DNA Polymerases', Nucleosides, Nucleotides and Nucleic Acids, 18: 3, 393 — 401
**To link to this Article: DOI:** 10.1080/15257779908043084
**URL:** http://dx.doi.org/10.1080/15257779908043084

NUCLEOSIDES & NUCLEOTIDES, 18(3), 393-401 (1999)

# SYNTHESIS OF A MONOCHARGED PEPTIDE NUCLEIC ACID (PNA) ANALOG AND ITS RECOGNITION AS SUBSTRATE BY DNA POLYMERASES

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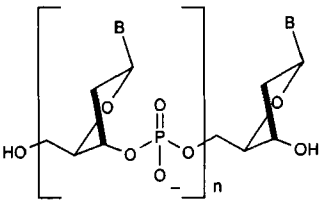
**ABSTRACT**: The preparation of a novel phosphoramidite monomer based on thyminyl acetic acid coupled to the secondary nitrogen of 2-(2-amino-ethylamino)ethanol is described. This monomer can be used to attach a deoxynucleotide to the carboxy terminus of a PNA oligomer by solid-phase synthesis. The resulting PNA primer is recognized as a substrate by various DNA polymerases.

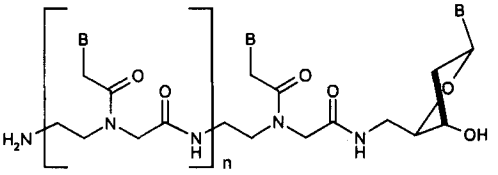
## INTRODUCTION

Peptide or Polyamide nucleic acids (PNAs)[1,2] are DNA mimetics in which the sugar-phosphate backbone has been replaced by an uncharged achiral polyamide backbone[3,4] consisting of N-(2-amino-ethyl)glycine units. PNAs specifically recognize complementary DNA or RNA forming DNA•PNA or RNA•PNA duplexes, respectively, which are more stable than their natural congeners. They therefore have the potential to replace oligonucleotides for use in antisense therapeutics[5] and diagnostics.[6] One limitation of PNAs is, however, that they are not recognized as substrates by nucleic acid processing enzymes, such as DNA polymerases.

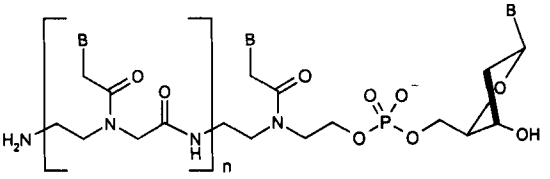
Recently, we have shown that uncharged PNA analogs having just one 5'-amino-5'-deoxynucleoside at their carboxy terminus (Scheme 1) can serve as primers of second strand synthesis on a DNA template using Klenow fragment (KF) of DNA Polymerase I



DNA primer



uncharged PNA primer **1**



monocharged PNA primer **2**

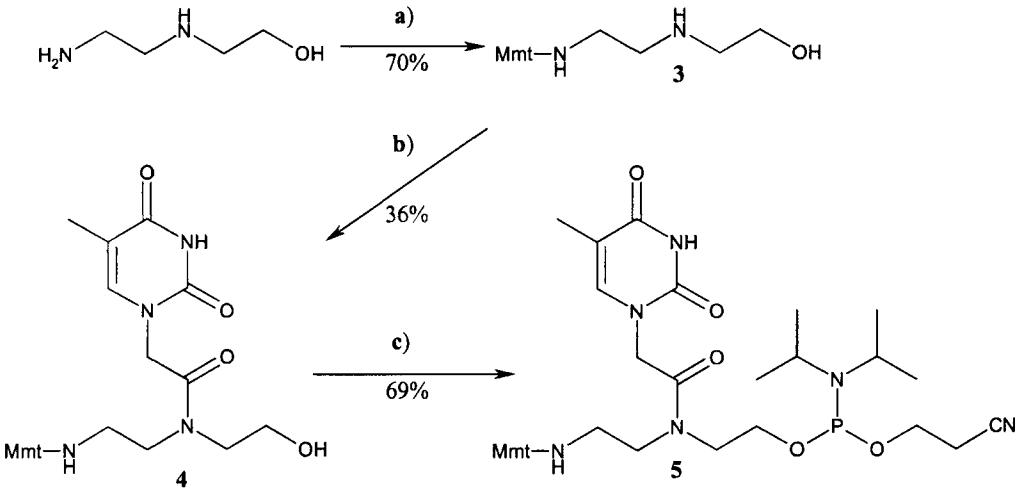
**SCHEME 1**: Chemical structure of DNA primer, uncharged PNA primer (**1**) and monocharged PNA primer (**2**).



(*Escherichia coli*) or *Vent* DNA Polymerase (*Thermococcus litoralis*).[7] However, for certain DNA polymerases examined in this study, the uncharged PNA analog turned out to be a poor substrate. Since crystallographic and biochemical studies suggested that binding of a primer complex to polymerases is mediated by interaction of the negatively-charged backbone with conserved amino acid residues in the polymerases, we were interested in more DNA-like "monocharged" PNA primers having a phosphodiester linkage between the 5'-position of the nucleoside and the uncharged PNA part (Scheme 1).

## RESULTS AND DISCUSSION

Synthesis of the monocharged PNA primer required the preparation of a novel phosphoramidite building block **5** (Scheme 2) which is based on commercially available 2-(2-amino-ethylamino)ethanol. The protecting groups of phosphoramidite monomer **5** are fully compatible with the Mmt (monomethoxytrityl) strategy used for the synthesis of PNAs[8] and PNA-DNA chimeras.[9-11] Reaction of excess 2-(2-amino-ethylamino)ethanol with Mmt-chloride gave predominantly the primary-amine protected aminoethylamino ethanol **3**. Thyminyl acetic acid[2] was then coupled to the secondary amine of **3** using




**SCHEME 2**: Reagents and conditions: a) Mmt-Cl, DMF; b) Thyminyl-$CH_2COOH$, TOTU, DIEA, DMF; c) $ClP(N(iPr_2))(OCH_2CH_2CN)$, DIEA, $CH_2Cl_2$



TOTU[12] as activator. Subsequent phosphitylation of the resulting thyminyl derivative **4** with 2-cyanoethyl-N,N-diisopropyl phosphoramidochloridite yielded the desired N-Mmt-protected phosphoramidite building block **5**.

The monocharged PNA primer **2** was synthesized starting from N-benzoyl-2'-deoxyadenosine-3'-O-succinate-derivatized CPG support. The phosphoramidite **5** was coupled to the 5'-hydroxy group of the CPG-bound deoxynucleoside using standard phosphoramidite oligodeoxynucleotide synthesis conditions except that double coupling (15 min each) was employed. After capping of unreacted nucleoside with acetic anhydride/N-methylimidazole the Mmt protecting group was removed with 3% trichloroacetic acid. PNA synthesis was then performed using Mmt protected monomers[13] as described previously.[8] The PNA primer **2** was cleaved from the support and deprotected by treatment with conc. ammonia, purified by semi-preparative C18 reversed phase HPLC and then characterized by MS (MALDI-TOF: m/z 4636.1; calculated 4635.41).

The ability of the novel monocharged PNA primer **2** to serve as substrate in primer extension reactions was then studied in comparison with a DNA primer and the previously described uncharged PNA primer **1**[7] using different DNA polymerases. The

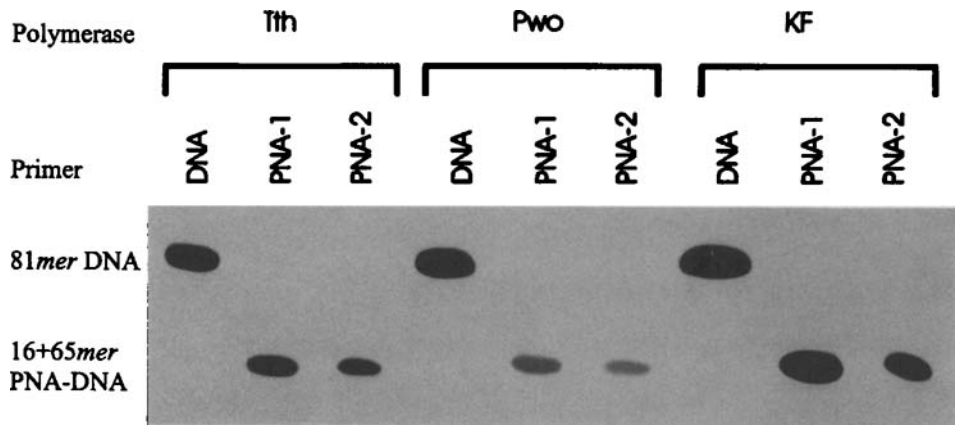



**FIGURE 1**: Primer extension reaction by *Tth*, *Pwo* and KF DNA polymerases using the DNA primer (lanes 1, 4, 7), the uncharged PNA primer **1** (lanes 2, 5, 8) and the monocharged PNA primer **2** (lanes 3, 6, 9).



primers were annealed to an 81-mer DNA template and the fill-in reactions were carried out in the presence of all four dNTPs (dCTP was replaced by [$\alpha$-$^{32}$P]-CTP) and various DNA polymerases. In Figure 1, an autoradiograph of denaturing polyacrylamide gel electrophoresis (PAGE) analysis of full-length products after fill-in reaction with *Tth*, *Pwo* and KF polymerases is depicted.

Evidently, the new monocharged PNA primer **2** can be elongated by all three DNA polymerases. Surprisingly, in all instances a weaker band is obtained for the full-length product when monocharged PNA primer **2** is used as compared to uncharged primer **1**. Both PNA primers are best accepted by KF, followed by *Tth* and *Pwo* DNA polymerases. Furthermore, PNA primer **2** was accepted by fewer of the polymerases examined than PNA primer **1** (Table 1). The most striking difference between the two primers is that PNA primer **2** is not accepted by reverse transcriptases whereas PNA primer **1** serves as substrate for reverse transcriptases from avian myeloblastosis virus (AMV) and moloney murine leukemia virus (M-MuLV). Although more extensive studies are necessary to obtain clear insight into the recognition of PNA primers in general, these data together with thermal denaturation studies[14] strongly suggest that the lower efficiency of PNA primer **2** compared to PNA primer **1** is due to stronger destabilization and structural perturbation of the duplex at the PNA-DNA junction.

**TABLE 1**: List of polymerases examined and their qualitative ability to elongate PNA primers

| **POLYMERASE** | **Ability to elongate** | |
|---|---|---|
| | PNA primer **1** | PNA primer **2** |
| Klenow fragment (KF) from *E. coli* | ++++ | +++ |
| T7 exo- (Sequenase™ 2.0) | - | - |
| *Thermus aquaticus* | - | - |
| *Thermus flavus* | - | - |
| *Thermus thermophilus* (*Tth*™) | +++ | ++ |
| *Thermus 'ubiquitos'* (HotTub DNA polymerase™) | - | - |
| *Pyrococcus furiosus* | - | - |
| *Pyrococcus woseii* | ++ | + |
| *Deep Vent™ exo-* | - | - |
| AMV-RT | + | - |
| M-MuLV-RT | + | - |



In conclusion, we have described the synthesis of the novel phosphoramidite **5** and its use in the synthesis of a PNA primer containing a C-terminal DNA nucleotide. The resulting monocharged PNA primer **2** can be extended on a DNA template by KF, *Tth* and *Pwo* DNA polymerases. Surprisingly, the more DNA-like phosphodiester linkage between the carboxy terminus of the PNA and the 5'-hydroxyl function of the nucleoside reduces the efficiency of primer extension compared to the uncharged PNA primer **1**. This is possibly due to incorrect spacing of the two terminal nucleobases. An potential alternative to the phosphodiester linkage described here would be the phosphonate linkage reported recently[14] which may be geometrically more favourable.

## EXPERIMENTAL SECTION

Materials and Methods: The following abbreviations are used: 4-Methoxytriphenylmethyl (Mmt); N,N-diisopropylethylamine (DIEA); N,N-dimethylformamide (DMF); O-[(Cyano(ethoxycarbonyl)methylen)amino]-1,1,3,3-tetramethyluronium-tetrafluoroborate (TOTU); trichloroacetic acid (TCA); controlled

pore glass (CPG™). Synthesis of PNA primer **1** ((pseudo-5')-taa tac gac tca cta (5'-amino)-T-3') has been described previously.[7] The 81-mer DNA template has the sequence 5'-GCC CCA GGG AGA AGG CAA CTG GAC CGA AGG CGC TTG TGG AGA AGG AGT TCA TAG CTG GGC TCC CTA TAG TGA GTC GTA TTA-3') and the DNA primer 5'-TAA TAC GAC TCA CTA T-3'. Upper case letters indicate DNA and lower case letters PNA.

*2-(2-(((4-Methoxyphenyl)-diphenylmethyl)-amino)-ethylamino)ethanol* (**3**): 2-(2-Amino-ethylamino)-ethanol (9.3g; 89.3mmol) was dissolved in anhydrous DMF (100ml) and cooled to 0°C. A solution of Mmt-Cl (9.24g; 29.9mmol) in $CH_2Cl_2$ (60ml) was added dropwise over 2h and the mixture was stirred for 16h at 4°C. The solvent was removed *in vacuo* and the residue was taken up in EtOAc (100ml) and water (50ml). The aqueous phase was extracted 4 times with EtOAc (40ml). The organic phase was dried ($Na_2SO_4$), filtered and evaporated *in vacuo*. The residue was purified by column chromatography on silica gel eluting with a gradient of 1-10% MeOH in EtOAc/$NEt_3$ (99:1, v:v) as the eluent. Yield 7.91g (70.3%). $R_f$ 0.14 (EtOAc/MeOH/$NEt_3$, 100:10:1, v:v:v); MS(FAB, MeOH/NBA/LiCl): 383.3 $(M+Li)^+$; $^1$H-NMR (d6-DMSO) δ 7.45-6.80, (14H, m, Mmt); 4.40 (1H, brs, OH); 3.75(3H, s, Mmt-$OCH_3$); 3.38(t+brs, $CH_2$+$H_2O$); 2.85 (2H, t, $CH_2$); 2.45(m, $CH_2$+DMSO); 2.05(2H, m, $CH_2$).



*N-(2-Hydroxyethyl)-N-(2-{[(4-methoxyphenyl)-diphenylmethyl]-amino}-ethyl)-2-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-acetamide* (**4**): N1-Carboxymethyl-thymine[2] (1.84g; 10mmol) was dissolved in anhydrous DMF (50ml), and TOTU (3.24g; 10mmol) and DIEA (3.4ml; 20mmol) were added. The mixture was stirred for 20 min and then compound **3** (3.76g; 10mmol) was added. The reaction was stirred for 16h at room temperature and evaporated *in vacuo*. The residue was dissolved in EtOAc (80ml) and extracted 4-times with water (20ml) containing 0.5% $NEt_3$. The organic phase was dried ($Na_2SO_4$), filtered and concentrated. The product was purified by chromatography on silica gel eluting with a gradient of MeOH (2-5%) in EtOAc/heptane (10:1, v:v) containing 1% $NEt_3$. Yield: 1.95g (36%) of an amorphous white solid. $R_f$ 0.46 (EtOAc/MeOH/ $NEt_3$, 100:20:1, v:v:v); MS($ES^+$): 543.3 $(M+H)^+$; $^1$H-NMR (d6-DMSO) δ 11.25 (1H, xs(rotamers), H-3); 7.45-6.80, (15H, m, Mmt+H-6); 4.80/4.60 (2H,

2xt(rotamers), $CH_2$); 4.68(2H, brs(rotamers), $CH_2$), 3.75(3H, s, Mmt-$OCH_3$); 3.60-3.20(m, $CH_2$+$H_2O$); 3.05/2.75 (2H, 2xt(rotamers), $CH_2$); 2.30-2.02(2H, m(rotamers), $CH_2$); 1.75(3H, 2xs(rotamers), T-$CH_3$).

*Diisopropyl-phosphoramidous acid 2-cyanoethyl ester 2-{(2-{[(4-methoxyphenyl)-diphenylmethyl]-amino}-ethyl)-[(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-acetyl]-amino}-ethyl ester* (**5**): Compound **4** (0.50g; 0.922mmol) was dissolved in anhydrous $CH_2Cl_2$ (15ml). DIEA (0.63ml; 3.69mmol) and 2-cyanoethyl-N,N-diisopropyl phosphoramidochloridite (0.24ml; 1.10mmol) were added to this solution. After 1h the solvent was evaporated *in vacuo*, the residue dissolved in EtOAc and washed four times with sat. aqueous NaCl. The organic phase was dried and evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using $CH_2Cl_2$/EtOAc/$NEt_3$ (49.5:49.5:1, v:v:v) as the eluent. Yield 0.47g (69%) yield. $R_f$ 0.45 ($CH_2Cl_2$/EtOAc, 1:1, v:v); MS (FAB): 743.4 ($M^+$); $^{31}$P-NMR ($CDCl_3$) δ 146.0, 146.7.



*(pseudo-5')-(Acetyl)-taa tac gac tca cta (eae(t)) A-3'* in which (eae(t)) is ethylamino-N-(thyminylacetyl)ethanol unit (uncharged PNA primer **2**): A synthesis column filled with 5'-O-dimethoxytrityl-N6-benzoyl-2'-deoxyadenosine-3'-O-succinate-derivatized CPG (5μmol) was attached to an Eppendorf Biotronik Ecosyn D300 DNA Synthesizer, and the 5'-terminal dimethoxytrityl group was removed by treatment with 3% TCA in $CH_2Cl_2$. Following washing with MeCN, a 0.1M solution of phosphoramidite **5** mixed with DNA activator solution (0.1 M tetrazole in MeCN) was applied to the column. The coupling reaction was run for 15 minutes, then repeated. After oxidation (iodine, pyridine, $H_2O$; 1min) and capping ($Ac_2O$, N-methylimidazole, lutidine in THF), the N-terminal Mmt group was removed with 3% TCA solution in $CH_2Cl_2$. PNA synthesis was then continued as described.[8] On completion of the synthesis the N-terminal Mmt group was removed, and the amino terminus was acetylated using with $Ac_2O$, N-methylimidazole, lutidine in THF. The PNA primer **2** was cleaved from the solid support using conc. $NH_4OH$ solution, and the base protecting groups were removed by heating this solution at 55°C for 5 hours. Following evaporation the oligomer was desalted on a Sephadex NAP-10 column (Pharmacia). Crude yield 69 $OD_{260}$. An aliquot of 34 $OD_{260}$ of crude product was purified by semi-preparative C18 reversed phase HPLC, eluting with 10-80% MeCN in

0.1M triethylammonium acetate. Following desalting on a Sephadex NAP-10 column and lyophilization, the PNA primer **2** was obtained in 5.1 $OD_{260}$ yield. MS (MALDI-TOF) m/z 4636.1 (calculated 4635.41).

Primer extension reactions: Enzymatic reactions were carried out in the appropriate reaction buffer (25 µl) containing 0.15 pmol primer / template complex and all required dNTPs at a final concentration of (a) 5 µM for thermolabile and (b) 50 µM for thermostable enzymes. dCTP was replaced by [$\alpha$-$^{32}$P]-dCTP (400 Ci/mmol). The reaction buffers were supplemented with $MgCl_2$ or $MgSO_4$ (2 mM) if required. Reactions were started by adding either 2 or 0.2 Units of the enzyme and incubated for 15 min either at 37 °C or 75 °C. Aliquots of each reaction (5 µl) were analyzed following heating for 20 min at 95 °C by denaturing (15% acrylamide, 35 W, 2 h) PAGE. Gels were fixed, dried and autoradiographed (PhosphorImager, Molecular Dynamics).



**ACKNOWLEDGEMENTS**: We thank S. Hein and D. Langner for excellent technical assistance, A. Peyman and S. Augustin for providing the DNA template, and A. Schäfer for measurement of the mass spectra. M.J.L. was supported by a scholarship from the German Academic Exchange Service in the program HSPII / AUFE.

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Received 11/18/98
Accepted 1/19/99