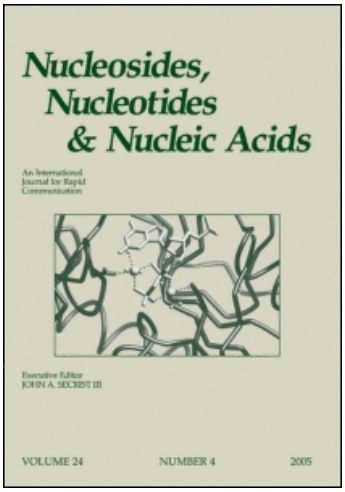

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IN VITRO INCORPORATION OF LNA NUCLEOTIDES

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□ *An LNA modified nucleoside triphosphate* **1** *was synthesized in order to investigate its potential to act as substrate for DNA strand synthesis by polymerases. Primer extension assays for the incorporation experiments revealed that Phusion High Fidelity DNA polymerase is an efficient enzyme for incorporation of the LNA nucleotide and for extending strand to full length. It was also observed that pfu DNA polymerase could incorporate the LNA nucleotide but it failed to extend the strand to a full length product.*

Keywords LNA nucleoside triphosphates; enzymatic incorporation

INTRODUCTION

Locked nucleic acid (LNA) is a general and versatile tool for high affinity recognition of ssDNA, ssRNA and dsDNA and has also been revealed as a most promising molecule for the development of oligonucleotide based therapeutics.[1] Moreover LNA oligomers have shown their significance in antisense studies both in vitro and in vivo.[2] In recent years, sugar modified nucleoside triphosphates and their incorporation into oligonucleotides have been a topic for many researchers. We now report the first attempts to incorporate LNA triphosphate **1** enzymatically into DNA.

Primer extension assays were performed by using DNA polymerases to incorporate LNA nucleotides in DNA strands and an appropriate primer and template sequences were designed (Table 1). The following enzymes were tested for LNA nucleotide incorporation: *Taq* DNA polymerase

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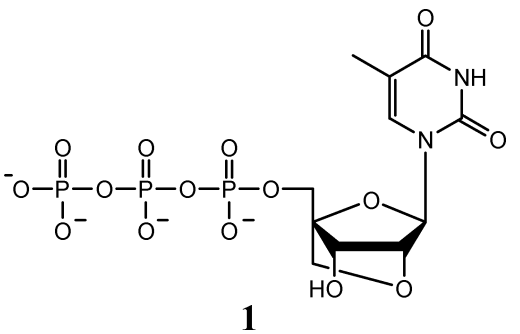
TABLE 1 Primer and template sequences used for the incorporation studies of **1** in this work

Incorporation of LNA-T, **1**	Length
Primer: 5′-TAATACGACTCACTATAGG-3′	19
Template:3′-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGG**A**T**A**G**A**CGG-5′	44

(Ampliqon, supplied by Bie & Bernsten A-S, Herlev, Denmark), Klenow Enzyme, large fragment (New England Biolabs, supplied by Medinova Scientific A/S, Glostrup, Denmark), T4 DNA polymerase (New England Biolabs, supplied by Medinova Scientific A/S, Glostrup, Denmark), *pfu* DNA polymerase (Invitrogen), *pfx* DNA polymerase (Invitrogen, A/S, Taastrup, Denmark), Speed STAR HS DNA polymerase (TaKaRa Bio, supplied by Cambrex Bio-Sciences aps, Copenhagen, Denmark), T7 RNA polymerase (Promega, GmBh, Mannheim, Germany), *E. coli* RNA polymerase (Sigma, Brøndby, Denmark), Phusion High Fidelity DNA Polymerase (Finnzymes, Supplied by Medinova Scientific A/S, Glostrup, Denmark), AMV reverse transcriptase (Finnzymes, supplied by Medinova Scientific A/S, Glostrup, Denmark) and mutant T7 R&DNA polymerase (Epicenter, Biotechnologies, Madison, WI, USA). Phusion High Fidelity DNA Polymerase was the only enzyme found to be efficient for incorporation of LNA nucleotides.

RESULTS AND DISCUSSION

The template sequence (44nt long) designed for the incorporation of compound **1** contains three incorporation sites for LNA building blocks at positions 37, 39, and 41. Primer extension assays show that Phusion High Fidelity DNA polymerase readily can extend the primer even after the incorporation LNA building blocks to full length (Figure 1). The fidelity of these experiments were checked by using one reaction set containing only dGTP, dCTP, and LNA-TTP in order to check the possible miss-incorporations (data not shown). Furthermore, the experiments were also monitored by



1

FIGURE 1 Structural representation of LNA-T nucleoside 5′-triphosphate.

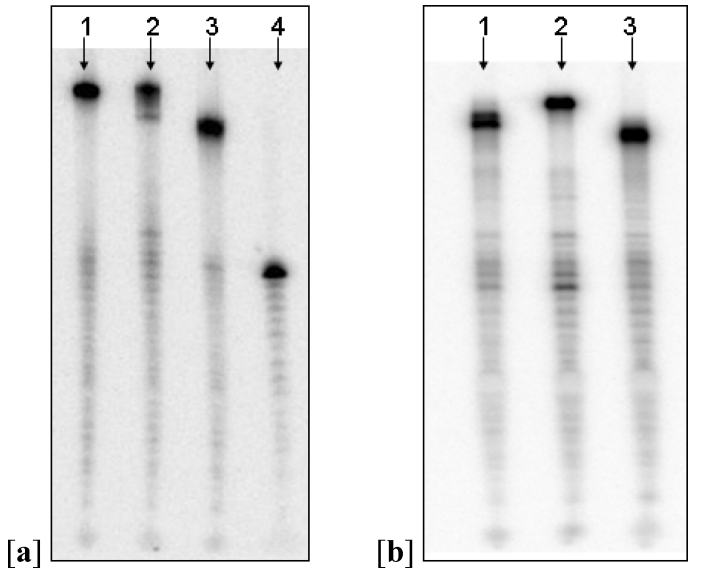


FIGURE 2 Enzymatic incorporation of LNA triphospahate 1 in DNA by [a] Phusion High Fidelity DNA polymerase: 1, positive control using all four natural dNTPs; 2, dTTP replaced with **1**, 3, negative control with no dTTP.; 4, primer; [b] *pfu* DNA polymerase: 1, dTTP replaced with **1**; 2, positive control using all four natural dNTPs; 3, negative control with no dTTP.

including a positive and a negative control. In the positive control reaction set, all four natural dNTP's were used and the primer was extended to full length (44nt long). In the negative control reaction set, dTTP, and LNA-TTP were absent and as expected this leads to extension only to the nucleotide before the first incorporation site (36nt long).

As shown in Figure 2b, *pfu* DNA polymerase could also incorporate LNA nucleotides but failed to extend to full length.

The reaction mixture was supplemented with $MnCl_2$ as Mn^{2+} is known to reduce the discrimination against different analogues with modifications in the furanose ring, the base or in the phosphate linkage.[3] Betaine enhancer solution (Purchased from Ampliqon) also was added as it is known to be an effective additive for templates which are extremely difficult to amplify. However, the addition of $MnCl_2$ and betaine enhancer solution was not mandatory for experiments performed by using *pfu* DNA polymerse.

CONCLUSION

We have synthesized LNA nucleoside triphosphate **1** in order to investigate their use as substrates for enzymatic oligonucleotide synthesis. Phusion High Fidelity DNA polymerase was found to be an efficient enzyme for the incorporation of LNA nucleotides in DNA strands and

afforded the full length product. Incorporation of LNA nucleotide **1** was also achieved by using *pfu* DNA polymerase, but further extension did not take place.

EXPERIMENTAL SECTION

Primer extension assays. The DNA primer sequences were purchased from DNA Technology and the template sequences from Sigma-Genosys. Phusion High Fidelity DNA Polymerase was purchased from Finnzymes. The primer sequences were 5′-^{32}P labelled by reaction with [γ-^{32}P]-ATP (~6000 Ci/mmol, Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs). The 5′-end labelled primers were annealed to the templates by combining primer and template in a molar ratio of 1:1 and heating the mixture to 80 °C for 2 min, followed by slow cooling to room temperature. The reaction mixtures were prepared in a total volume of 20 μL by adding 0.6 μL of a solution containing 5′-^{32}P labelled primer-template (5 pmol) complex, 4 μL of 5× Phusion HF buffer (included in the Phusion High Fidelity DNA Polymerase Kit, Finnzymes), 1 μL of $MnCl_2$ (50 mM), 1 μL of betaine enhancer solution (2 M, Ampliqon), 1 μL of dNTP mixture (10 mM in each dNTP; for the preparation of nucleoside triphosphate mixtures containing LNA 5′-triphosphates, 100 mM of LNA triphosphates were used), 1 μL of Phusion High Fidelity DNA Polymerase (2U/μL), and 11.6 μL of distilled water (2 times distilled). The reaction mixtures were gently vortexed and heated at 72°C for 2 hours. The polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 20 mM EDTA). Analysis was performed by gel electrophoresis for 30 minutes on a 13% 7 M urea polyacrylamide gel in the presence of a TBE buffer (100 mM Tris, 90 mM Boric acid, 1 mM EDTA) of pH 8.4. Products were visualized by phosphor imaging.

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