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Towards the enzymatic synthesis of phosphorothioate containing LNA oligonucleotides

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Abstract

Therapeutic oligonucleotides require the addition of multiple chemical modifications to the nucleosidic scaffold in order to improve their drug delivery efficiency, cell penetration capacity, biological stability, and pharmacokinetic properties. This chemical modification pattern is often accompanied by a synthetic burden and by limitations in sequence length. Here, we have synthesized a nucleoside triphosphate analog bearing two simultaneous modifications at the level of the sugar (LNA) and the backbone (thiophosphate) and have tested its compatibility with enzymatic DNA synthesis which could abrogate some of these synthetic limitations. While this novel analog is not as well tolerated by polymerases compared to the corresponding α -thio-dTTP or LNA-TTP, α -thio-LNA-TTP can readily be used for enzymatic synthesis on universal templates for the introduction of phosphorothioated LNA nucleotides.

Keywords

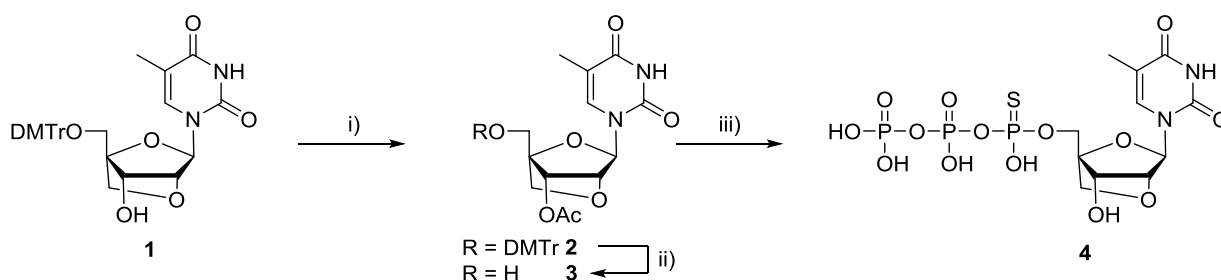
Locked nucleic acids; phosphorothioates; modified nucleotides; polymerase synthesis; Terminal deoxynucleotidyl transferase (TdT)

The recent advent of mRNA-based vaccines^{1, 2} and the FDA-approval of several oligonucleotide drugs³ clearly underscore the importance and relevance of therapeutic oligonucleotides. Regardless of their mode of action (gene silencing, antisense mechanisms, catalytic hydrolysis...), the main (partially solved) challenges associated with therapeutic oligonucleotides include efficient drug delivery to specific targets, chemical toxicity and undesired side-effects, as well as rapid nuclease-mediated degradation.^{3, 4} These limitations are often addressed by endowing oligonucleotides with chemical modifications either at the level of internal nucleotides or at their termini using conjugation chemistry. In this context, the sugar and phosphate backbone are popular sites for the introduction of chemical modifications to improve the stability, the pharmacokinetic and pharmacodynamic profiles as well as the drug delivery efficiency of oligonucleotides. The incorporation of phosphorothioate (PS) units in the phosphate backbone, where one of the non-bridging oxygens of the phosphodiester linkages is substituted with a sulfur atom, results in the formation of P-S bonds which are more

resistant against the activity of nucleases compared to the parent P-O linkages.⁵ In addition to increased stability, PS-containing oligonucleotides bind to plasma proteins including albumin as well as heparin-binding proteins which improves their pharmacokinetic profiles.⁶ However, the replacement of an oxygen with a sulfur atom introduces a chiral center at the level of the phosphorous atom (Sp and Rp diastereomers)⁷ which in the absence of stereocontrolled synthesis will generate 2^{N-1} diastereomers in a single N-mer oligonucleotide which might have different physicochemical, pharmaceutical and structural properties.⁸

Besides backbone modifications such as PS, sugar modifications are included in most therapeutic oligonucleotides mainly to enhance their serum stability as well as their affinity and specificity for target mRNA. A prime example of sugar modifications are the bridged nucleic acids where the sugar pucker is locked in a 3'-*endo*/*N*-type configuration to minimize entropic losses upon hybridization to target sequences. One of the most used variation is LNA (Locked Nucleic Acids) where an additional methylene unit connects the 4'-carbon to the 2'-oxygen of the ribose.⁹ LNA nucleotides can massively stabilize duplexes since impressive increases in T_m values of 2-10°C per modification can be observed which is important for target recognition and specificity, particularly in the antisense strategy. Oligonucleotides equipped with LNA modifications are also more resistant to nuclease-mediated degradation and are thus often integrated in therapeutic oligonucleotides.¹⁰⁻¹² Most antisense and related therapeutic oligonucleotides contain different combinations of sugar modified nucleotides (e.g. 2'-OMe and LNA) and PS units which improve their efficiency and drug delivery capacities.¹³⁻¹⁶ On the other hand, the inclusion of multiple types of modifications comes at the expense of synthetic efforts. Herein, we questioned the possibility of using a single modified nucleoside triphosphate to introduce concomitantly sugar (LNA) and backbone (PS) modifications. Such an approach can be compatible with the synthesis of long sequences¹⁷ or even genes as well as controlled enzymatic synthesis of modified therapeutic oligonucleotides.^{18, 19} We also envisioned that using polymerase-mediated synthesis might allow some degree of stereocontrol since polymerases generally prefer alpha-S-thiotriphosphates over the corresponding alpha-R-thiotriphosphates as substrates and yield the corresponding Rp-containing oligonucleotides.²⁰

In order to address these questions, we first synthesized α -thio-LNA-TTP **4** which consists of an LNA nucleoside equipped with an α -thiotriphosphate moiety (Scheme 1). To do so, we converted 5'-DMTr-protected LNA-T nucleoside **1** into a suitably protected 3'-OAc analog **3** by application of standard protocols.^{21, 22} We modified a 4 step-1 pot protocol originally developed by Ludwig and Eckstein²³ where we trapped the cyclic triphosphate intermediate with the Beaucage reagent²⁴ to obtain the desired modified nucleotide **4** in good yields (31% over 4 steps).



Scheme 1. Synthetic route to the modified LNA triphosphate **4**. Reagents and conditions: i) Ac_2O , DMAP, pyridine, rt, 1h, 91%; ii) TFA, DCM, rt, 1h, 80%; iii) a) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; b) $(n\text{Bu}_3\text{NH})_2 \text{H}_2\text{P}_2\text{O}_7$, DMF, $n\text{Bu}_3\text{N}$, rt, 45 min; c) Beaucage reagent, pyridine, rt, 30 min; d) NH_3 (aq.), rt, 2h, 31% over 4 steps.

We next evaluated the compatibility of α -thio-LNA-TTP **4** with polymerase assisted DNA synthesis. We first turned our attention to the terminal deoxynucleotidyl transferase (TdT) since this polymerase

is currently evaluated to serve in controlled enzymatic DNA synthesis which holds promises to complement or even rival phosphoramidite-based synthetic approaches.²⁵⁻²⁹ To do so, we carried out TdT-mediated tailing reactions using 19 nucleotide long primer **P1**³⁰ (see Supporting Information) with α -thio-LNA-TTP **4** and different metal cofactors (Figure 1) and compared the efficiency of the tailing reactions with those obtained either with unmodified LNA-TTP (synthesized by application of known protocols³¹) or with α -thio-dTTP (Figures S1 and S2, respectively). As reported previously, LNA-TTP is well recognized by the TdT polymerase and near quantitative conversion of the primer to the corresponding n+1 product can be obtained (Figure S1).³¹ However, irrespective of the experimental conditions, no second incorporation of an LNA nucleotide was observed, as reported previously for this nucleotide as well as for other LNA nucleotides bearing small chemical modifications.³¹⁻³³ On the other hand, α -thio-dTTP is an excellent substrate for the TdT polymerase since the expected patterns (i.e. small product dispersities and large size averages) are formed which are comparable to those of unmodified or some modified nucleoside triphosphates and with near full consumption of primer **P1** (Figure S2).³⁰ Surprisingly, α -thio-LNA-TTP **4** is not a very good substrate for the TdT polymerase unlike the related LNA-TTP and α -thio-dTTP, since n+1 product formation could be observed but only in low yields (~50%) irrespective of the experimental conditions and the nature of the metal cofactor (Figure 1).

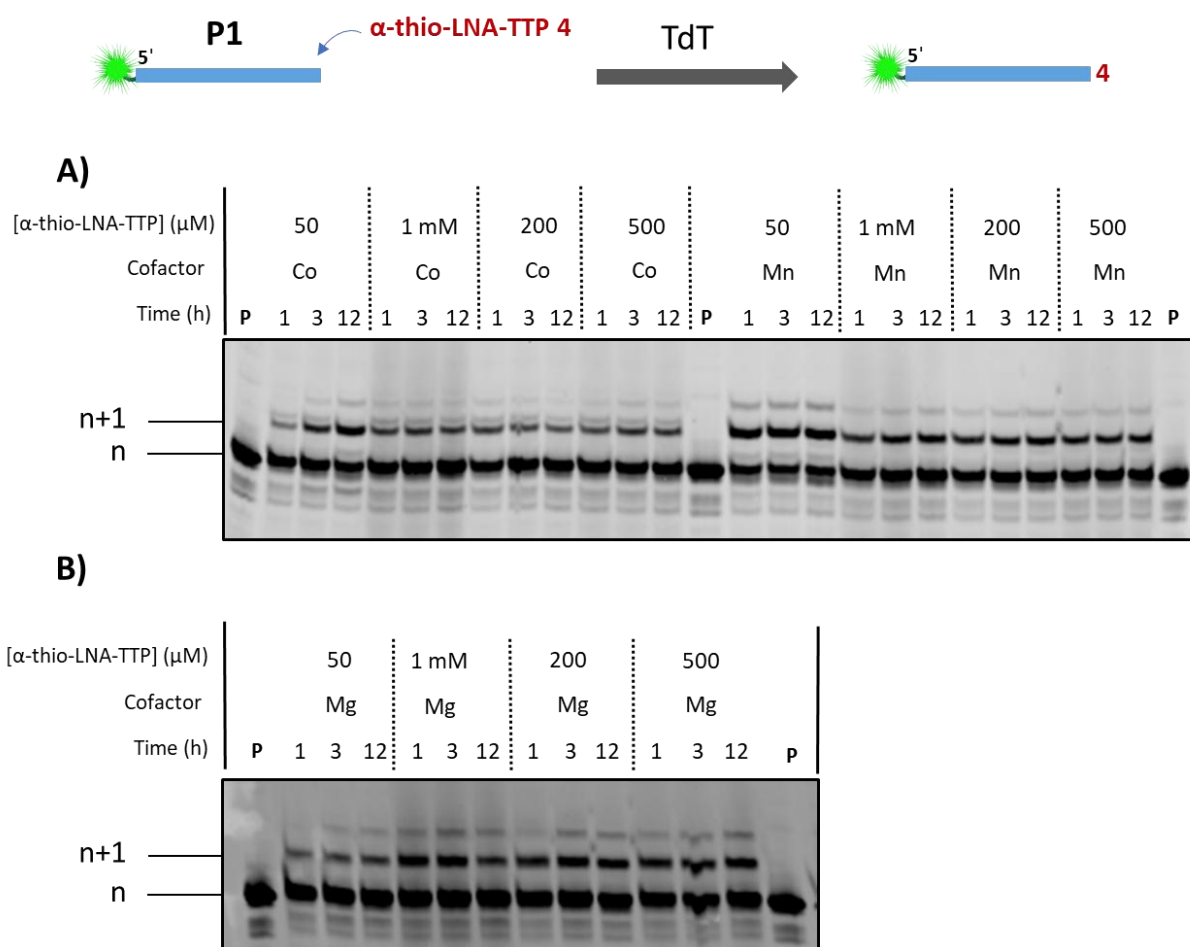


Figure 1. Gel images (PAGE 20%) of TdT-mediated tailing reactions carried out with 10 U of polymerase, primer **P1**, varying concentrations of α -thio-LNA-TTP **4** and reaction times, with different metal cofactors (A) 0.25 mM Co^{2+} or 1 mM Mn^{2+} , or B) 1 mM Mg^{2+}), and at 37°C. **P** represents unreacted primer.

In order to rationalize these surprising findings, we undertook an Autodock simulation study using the reported 2.6 Å resolution X-ray structure of the ternary complex of mouse TdT with ssDNA and an incoming nucleotide (PDB 4I27).³⁴ In each analysis, we replaced the incoming triphosphate present in the active site of the polymerase with either *Rp*-5'-thio-LNA-TTP (Figures 2A and S3A), *Sp*-5'-thio-LNA-TTP (Figures 2B and S3B), LNA-TTP (Figure S4), *Rp*-5'-thio-dTTP (Figure S5), and *Sp*-5'-thio-dTTP (Figure S6). A careful inspection of the binding pockets and the calculated free energies of binding revealed that both diastereomers of 5'-thio-LNA-TTP fitted well within the active site of the TdT polymerase with comparable free energies (-15.27 kcal/mol for *Rp* and -15.49 kcal/mol for *Sp*). However, while the α -phosphorous of *Rp*-diastereomer is perfectly aligned for the nucleophilic attack of the 3'-OH of the oligonucleotide, such an orientation is not observed in the case of the *Sp*-diastereomer suggesting that *f* does not act as a good substrate for the TdT polymerase. Such a stereopreference has been observed previously when homochiral primers were used in conjunction with the TdT polymerase since *Sp*-containing sequences did not yield efficient tailing reactions.³⁵ In the case of the parent LNA-TTP, a favorable binding energy (-17.90 kcal/mol) and orientation of the α -phosphorous moiety suggest that this modified nucleotide is accepted as a substrate by the polymerase as reflected by the efficient incorporation assays (Figure S1).³¹ Lastly, both diastereomers of 5'-thio-dTTP present favorable binding energies comparable to those calculated for α -thio-LNA-TTP **4** (-15.50 kcal/mol for the *Rp*-diastereomer and -15.53 kcal/mol for the *Sp*-diastereomer) and the α -phosphorous centers are aligned for the in-line attack of the nucleophile. This docking analysis as well as TdT-tailing reactions performed with racemic mixtures of 5'-thio-dTTP (Figure S2) do not allow to conclude on the stereospecificity of the TdT on incoming triphosphates.

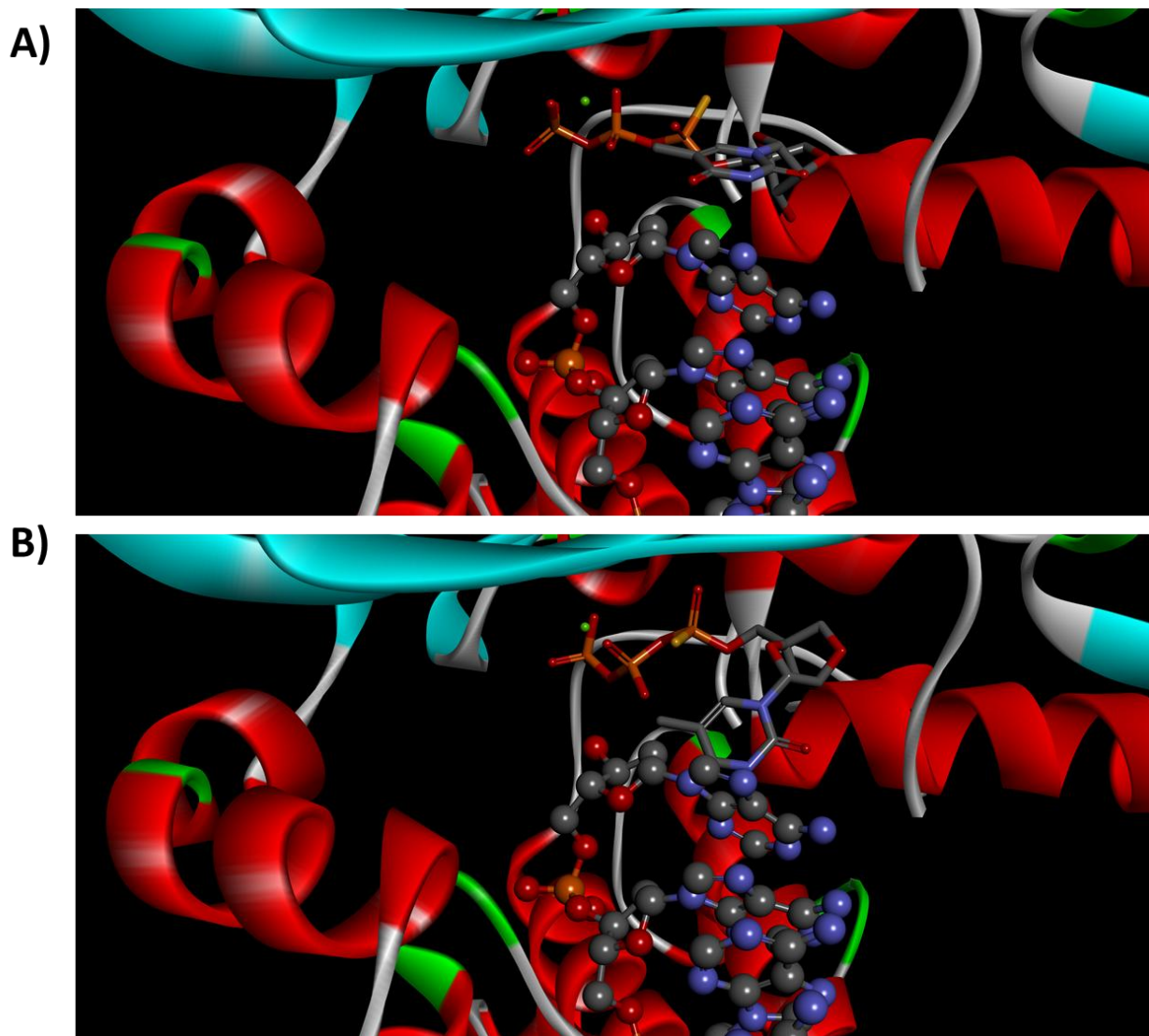


Figure 2. Docking results obtained with A) Rp-5'-thio-LNA-TTP and B) Sp-5'-thio-LNA-TTP and the binary complex of mouse TdT and a ssDNA primer (PDB 4I27).

Next, we sought to evaluate the possibility of using α -thio-LNA-TTP **4** under primer extension (PEX) reaction conditions either using a 31-nucleotide long template **T1**³⁶ (Figure 3) or a universal template **T2** designed for controlled DNA synthesis (Figure 4).³⁷ To do so, PEX reactions were carried out with different polymerases and by using either α -thio-LNA-TTP **4** or α -thio-dTTP instead of the natural counterpart dTTP. When template **T1** was used, only Therminator was capable of extending the 15-mer primer **P2** to full length products and all other polymerases stalled after the incorporation of a first phosphorothioate-modified LNA nucleotide (Figure 3A). On the other hand, all polymerases readily accepted α -thio-dTTP as a substrate and produced the expected full length products with full conversion of primer **P2** (Figure 3B). We then carried out PEX reactions using the universal template **T2** that contains five 3'-terminal nucleotides consisting of a mixture of all four nucleotides (for a total of 1024 combinations). Hence, under PEX reaction conditions, primer **P2** should be extended by five additional nucleotides regardless of the nature of the nucleobase on the incoming nucleoside triphosphate. Indeed, in the presence of all four natural dNTPs, full extension products can be observed (T+ lanes in Figure 4). We observed full conversion of primer **P2** into the n+2 product when Therminator and Vent (*exo*⁻) were used as polymerases together with α -thio-LNA-TTP **4** as substrate

(Figure 4A). Other polymerases including HemoKlenTaq and *Bst* produced a distribution of n , $n+1$, and $n+2$ products although some nucleolytic degradation could be observed in these reactions. All other polymerases were rather reluctant to use α -thio-LNA-TTP **4** as substrate. Also in this setting, α -thio-dTTP appears to be a better substrate for polymerases than α -thio-LNA-TTP **4** since full length ($n+5$) products could be observed with Terminator, Vent (*exo*), and HemoKlenTaq. In addition, all other polymerases accepted α -thio-dTTP as a substrate to various degrees since product distributions ranging from $n+1$ to $n+4$ could be observed (Figure 4B).

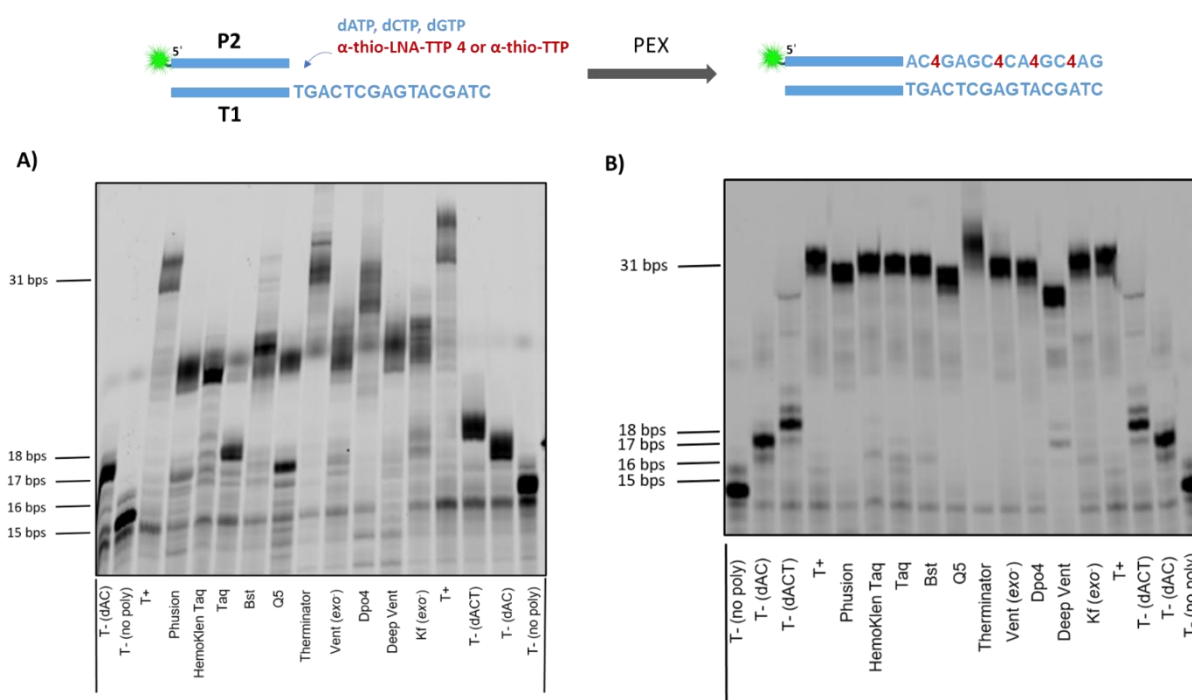


Figure 3. Gel image (PAGE 20%) analysis of products stemming from PEX reactions with primer **P2** and template **T1** and in the presence of various polymerases and either A) α -thio-LNA-TTP **4** (200 μ M) or B) α -thio-dTTP (200 μ M). Reactions were carried out with 10 U of polymerases and for 1h. T+ represents a positive control reaction with all four natural dNTPs, T-(dACT) and T-(dAC) represent negative controls run with only three and two natural nucleotides, respectively, and T- (polymerase) represents a control reaction run in the absence of polymerase.

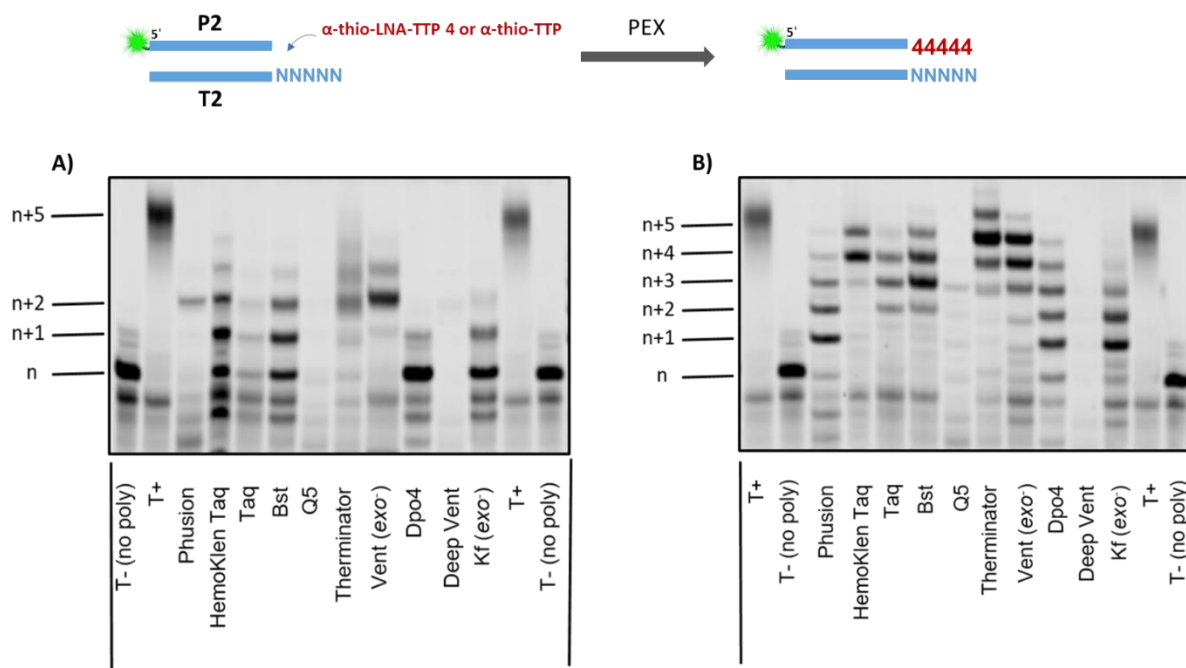


Figure 4. Gel image (PAGE 20%) analysis of products stemming from PEX reactions with primer **P2** and template **T2** and in the presence of various polymerases and either A) α -thio-LNA-TTP **4** (200 μ M) or B) α -thio-dTTP (200 μ M). Reactions were carried out with 10 U of polymerases and for 1h. T+ represents a positive control reaction with all four natural dNTPs, and T-(polymerase) represents a control reaction run in the absence of polymerase.

In conclusion, we have devised a high yielding synthetic pathway for the simultaneous incorporation of chemical modifications into the scaffold of nucleoside triphosphates which can readily be expanded to other analogs. This is of importance since only few, mainly TNA-based, examples of nucleotides bearing modifications at more than one specific location have been reported so far.^{33, 36, 38-44} We then investigated the possibility of using α -thio-LNA-TTP **4** either in TdT-mediated tailing reactions for future use in controlled DNA synthesis or in PEX reactions to generate small therapeutic oligonucleotides or larger modified fragments. Surprisingly, α -thio-LNA-TTP is not very well tolerated by polymerases unlike nucleotides bearing similar but only one modification (i.e. LNA-TTP or α -thio-dTTP). In order to rationalize these findings, we have performed docking studies with different modified nucleotides and a primer-TdT complex which hint at a possible stereopreference of the enzyme for Rp-5'-thio-LNA-TTP. However, in order to fully address a possible stereopreference and investigate that of natural 5'-thio-dNTPs, stereospecific syntheses of each diastereomer would be required.⁸ Lastly, PEX reactions with a universal template nonetheless demonstrate that α -thio-LNA-TTP **4** can be used with some polymerases for the enzymatic construction of short, modified oligonucleotides.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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