Benzoyl and Pivaloyl as Efficient Protecting Groups for Controlled Enzymatic Synthesis of DNA and XNA Oligonucleotides

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Benzoyl and pivaloyl as efficient protecting groups for controlled enzymatic synthesis of DNA and XNA oligonucleotides

Marie Flamme,[a] Dace Katkevica,[b] Karlis Pajuste,[b] Martins Katkevics,[b] Nazarii Sabat,[a] Steven Hanlon,[c] Irene Marzuoli,[c] Kurt Püntener,[c] Filippo Sladojevich,[d] and Marcel Hollenstein,[*,a,]

Abstract

Controlled enzymatic synthesis is an alluring alternative to solid-phase synthesis and polymerase-mediated incorporation of nucleotides for the crafting of chemically modified, therapeutic oligonucleotides. While this approach has met some success for the elaboration of long, unmodified DNA sequences, very little research efforts have been dedicated to xeno nucleic acids (XNAs). Here, we have evaluated the possibility of using various 3'-*O*-blocking groups for controlled synthesis of locked nucleic acids (LNA). LNA nucleosides were equipped with protecting groups used in synthetic organic chemistry and were evaluated for their stability. The most promising candidates, benzoyl- and pivaloyl-protected nucleosides, were converted to the corresponding nucleotides. The resulting modified nucleotides were shown to be accepted by various polymerases. While single incorporation events were observed in high yields, strong esterase activity of polymerases represents a lasting hurdle that needs to be overcome. Overall, this article represents an additional step towards the controlled enzymatic synthesis of LNA-containing oligonucleotides and could be extended to other sugar or nucleobase modified nucleotides.

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Introduction

Synthetic biology and organic synthesis have produced numerous chemical alternatives to natural DNA and RNA which support development of therapeutic and diagnostic applications.[1] For instance, the introduction of chemical modifications into oligonucleotides stabilizes the nucleosidic scaffold against nuclease mediated degradation and enhances the therapeutic efficacy of nucleic acids.[2] Similarly, nucleobase and sugar modifications are compatible with Darwinian evolution methods and yield binders (aptamers) and catalysts (DNAzymes and ribozymes) with remarkable properties that are often unrivalled by unmodified counterparts.[2d, 3] Lastly, modified building blocks represent key elements of recently commercialized mRNA-based vaccines in response to the SARS CoV2-pandemic where they improve translation into target antigens and avoid detection by pattern recognition receptors.[4] So far, synthetic access to chemically modified nucleic acids is granted either by automated solid-phase synthesis using phosphoramidite building blocks[5] or by the enzymatic polymerization of nucleoside triphosphates.[6] Solid phase synthesis is particularly adapted for large scale production of chemically modified nucleic acids and particularly of xenonucleic acids (XNAs) which consist of oligonucleotides containing different sugar chemistries that convey them a high degree of orthogonality to canonical nucleic acids.[7] However, such an approach is restricted to rather short sequences and not all functional groups are tolerant to the rather harsh conditions imposed during the different steps of a synthetic cycle. Similarly, the enzymatic incorporation of nucleotide analogs is a versatile and mild method for the generation of modified oligonucleotide of any size and is particularly suited for SELEX (Systematic Evolution of Ligands by EXponential enrichment)[8] and related methods of *in vitro* selection of functional nucleic acids. On the other hand, substituting natural for modified nucleotides might lead to an over-saturation of functional groups which might be detrimental to mediating efficient binding or catalytic activity. Also, this approach requires the modified nucleotides to be accepted as substrates by polymerases which is not always the case, particularly for XNAs such as LNA (locked nucleic acids).[9] Hence synthesizing long oligonucleotides with modifications located at well-defined positions within the sequence remains challenging.[10] In this context, controlled enzymatic synthesis of DNA represents an alternative approach that is gaining momentum. In this method, nucleoside triphosphates (dNTPs) are equipped with transient blocking groups which are incorporated, mainly by template independent DNA polymerases, into growing DNA sequences immobilized on a solid support.[3g, 11] The protecting groups can either be placed on the nucleobase[12] or at the level of the 3'-position of the deoxysugar[13] moiety where they serve as reversible chain terminators. After incorporation of the modified nucleotide, the protecting groups are removed and the extended primer is available for the next synthetic cycle. Successful example of nucleotides equipped with reversible blocking groups have been reported and used for the de novo synthesis of DNA. However, controlled enzymatic synthesis has been barely explored for alternate sugar chemistries, particularly of XNAs.[14] Here, we have evaluated the possibility of using different reversible protecting groups for controlled enzymatic LNA synthesis. Among the various terminators, benzoyl and pivaloyl protecting groups appear to display interesting properties since the corresponding nucleotides are well-tolerated by polymerases and single incorporation events are achieved in high yields. This article thus sets a new step towards the development of methods for the controlled enzymatic synthesis of LNA containing oligonucleotides.

Results and discussion

Design and synthesis of 3'-*O*-protected LNA nucleosides

The design of LNA nucleotides equipped with reversible terminators involves a fine balance of multiple structural and conceptual elements. First, we opted to incorporate the reversible blocking groups at the level of the 3'-*O*-position of the sugar rather than on the nucleobase. Indeed, incorporation of bulky residues on the nucleobase often comes at the expense of a molecular scar (i.e. a chemical functionality left on the nucleobase due to incomplete removal of the linker arm) which remains after removal of the transient protecting group. While such an approach is fully compatible with sequencing-by-synthesis methods,[12, 15] these molecular scars may negatively affect the properties of the resulting functional or therapeutic nucleic acids. Second, the protecting group must be stable upon storage of the nucleotides as well as during the enzymatic reaction step to ensure the strict incorporation of one single nucleotide during each synthetic cycle. Hence, the protecting group needs to be stable under higher pH (i.e. 8.0-9.0) conditions which are required for optimal polymerase activity and exposure to elevated temperatures (ranging from 37°C to 65°C depending on the nature of the polymerases). In addition, the blocking group needs to be resistant against the intrinsic esterase[16] and phosphatase[14, 17] activity displayed by a number of DNA polymerases. Third, removal of the blocking group should proceed in high yields and involve mild conditions that do not damage the nascent DNA/LNA chain. Lastly, the reversible moiety should not interfere with residues located in the active site of polymerases so that the nucleotide is compatible with enzymatic synthesis. Based on these elements as well as results published by our laboratory[9a, 14] and by others,[3g, 13b, d, 16c, 18] we envisioned to use several potential protecting groups on LNA-T nucleosides (Figure 1). Indeed, we considered a series of more robust ester functionalities including benzoyl (**1**), *o*-nitrobenzoyl (**2**), allyl (**3**), and pivaolyl (**4**), as well as a carbonate (**5**) and an ether functionality (**6**) used in synthetic organic chemistry as standard protecting groups.

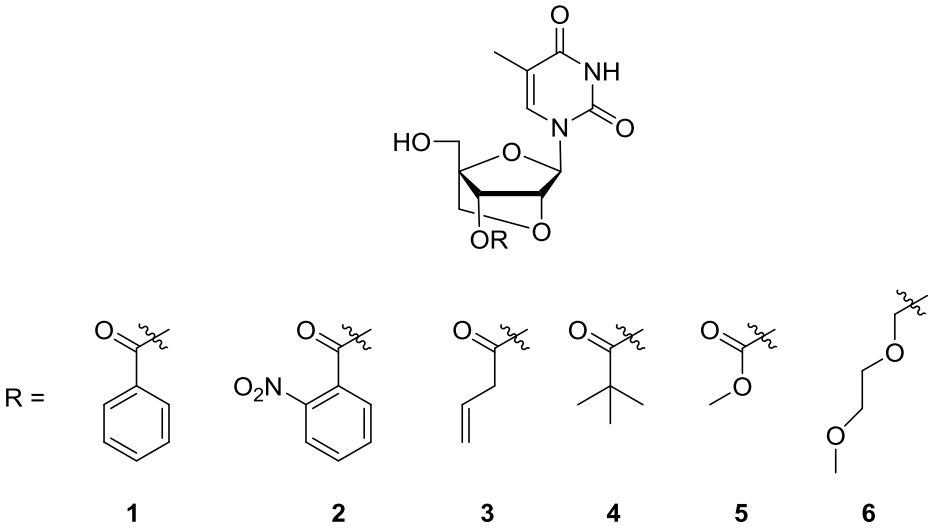


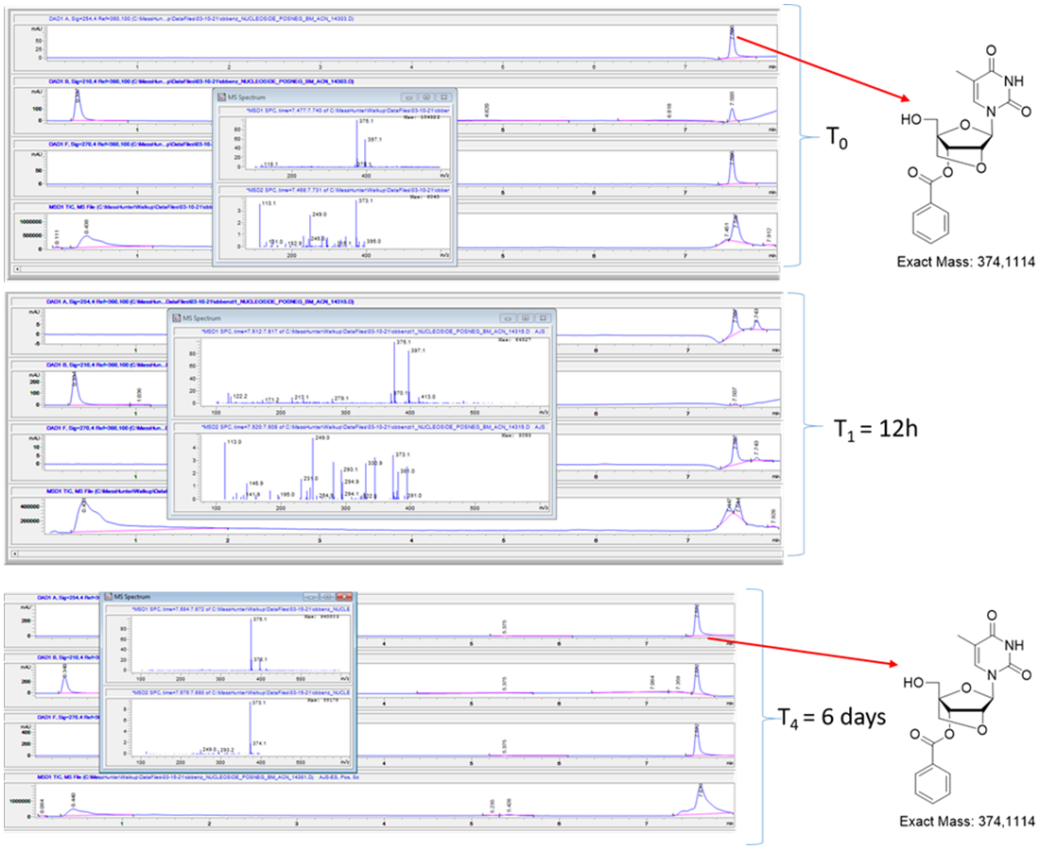
Figure 1. Chemical structures of LNA nucleosides equipped with 3'-*O*-blocking groups considered in this study.

After these considerations, we synthesized the 3'-*O*-modified LNA nucleoside analogs starting from the 5'-DMT-*O*-protected LNA-T nucleoside obtained by application of literature protocols[9a, 19] and with activated ester, carbonate, and ether precursors (see Schemes S1-S6 and the Supporting Information). The resulting protected intermediates were then treated under mild acidic conditions to remove the trityl blocking groups to yield compounds **1**-**6** in good overall yields (Supporting Information).

Stability assay of nucleosides and synthesis of nucleoside triphosphates

With analogs **1**-**6** at hand, we evaluated whether the protecting groups affixed at position 3' would resist incubation in the reaction buffer of the Terminal deoxynucleotidyl Transferase (TdT). This polymerase is often used in the context of controlled enzymatic synthesis due to its capacity at catalyzing the template-independent appendage of nucleotides at the 3'-termini of single stranded DNA oligonucleotides.[3g, 11a, 13d, 20] In addition, the typical reaction conditions used for this polymerase (i.e. 37°C, pH = 7.9, and Co^{2+} as cofactor) are rather mild and hence will also give a good indication of whether a nucleoside/nucleotide analog is stable upon longer storage. Such an analysis reduces the synthetic and purification burden imposed by nucleoside triphosphate synthesis and allows to identify protecting groups that are stable upon storage and in reaction buffer. Hence, we incubated the different nucleosides in TdT buffer 1X supplied with the enzyme for given amounts of time and analyzed the resulting products by LCMS (Figure 2 and Figures S1-S4, Supporting Information). Nucleosides **1** and **4** equipped with benzoyl and pivaloyl remained unaltered upon incubation and no deprotected LNA nucleoside could be observed even after 6 days of incubation (Figure 2). On the other hand, nucleosides equipped with a nitrobenzyl and an allyl-ester moiety (nucleosides **2** and **3**) as well as nucleoside **5** protected with a methyl-carbonate group appear to be prone to hydrolysis (Figures S1-S3) since formation of free LNA nucleoside can already be observed after 12h of incubation and at least 50% of the starting material is converted to deprotected LNA upon longer incubation times (24h or 6 days). Surprisingly, LNA-nucleoside **6** equipped with a 3'-*O*-MEM group is the least stable of all tested nucleosides since full conversion to the unprotected, parent nucleoside can be observed after only 1h of incubation in TdT buffer (Figure S4). In depth mechanistic investigations would be required to understand the poor stability of such an ether moiety but presumably the rather high [Mg^{2+}] present in the buffer promotes hydrolysis of the ether blocking group via coordination to the oxygen atom(s) of the MEM ether.

A)


T0
T1 = 12h
T4 = 6 days
Exact Mass: 374,1114
Exact Mass: 374,1114

B)

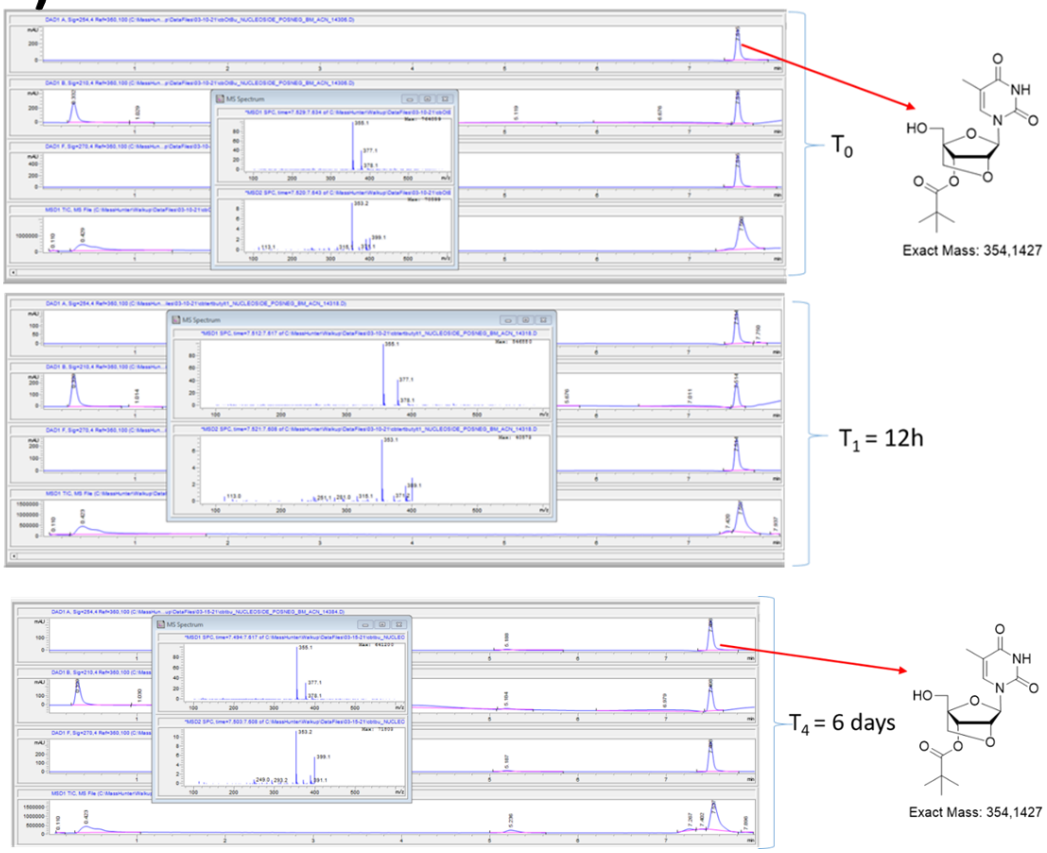
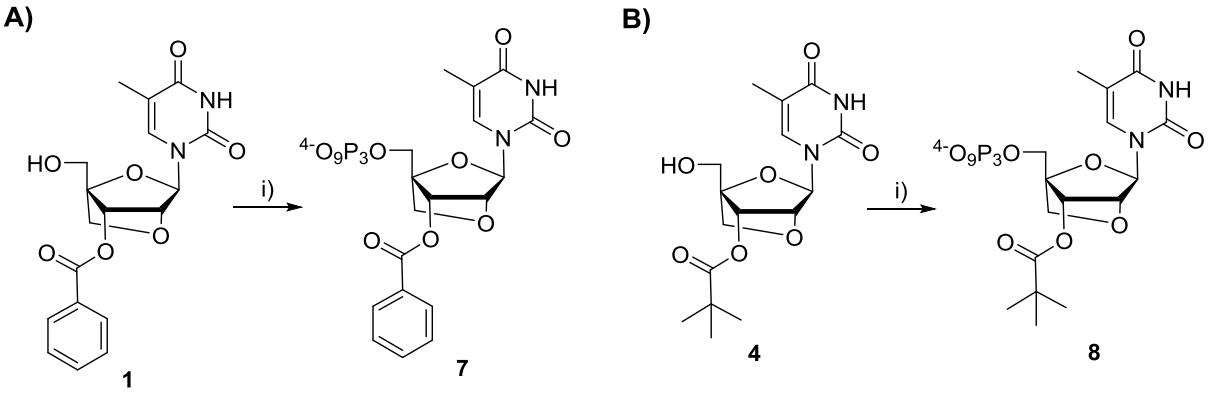

T0
T1 = 12h
T4 = 6 days
Exact Mass: 354,1427
Exact Mass: 354,1427

Figure 2. A) LCMS profile of nucleoside analog **1** and B) LCMS profile of nucleoside **4** incubated in TdT buffer 1X at 37°C during 0h, 12h, and 6 days. Nucleosides were at 20 mM concentration. For each condition, LC traces were recorded for UV absorptions at 254 nm, 210 nm, and 270 nm, respectively. The fourth plot represents the total ion current (TIC) chromatograms. The inserts show the deconvoluted mass spectrum. For close-up views see Figures S42-S45.

Having established that benzoyl- and pivaloyl-ester protecting groups can resist hydrolysis during long term storage and prototypical enzymatic reaction conditions, we performed docking experiments with the corresponding nucleotides to evaluate whether the protected nucleotides might be compatible with enzymatic synthesis. In these Autodock simulations[21] we have used the previously reported X-ray structure of the ternary complex of mouse TdT in conjunction with a ssDNA oligonucleotide and an incoming nucleotide (PDB 4I27).[22] In both simulations, we have substituted the incoming nucleotide with pivaloyl- and benzoyl-3'-*O*-protected LNA-TTP analogs (Figures S5 and S6, Supporting Information). The binding energies for both nucleotides in the active site of the TdT polymerase were highly favorable (-18.19 kcal/mol and -16.53 kcal/mol for the pivaloyl and the benzoyl protected nucleotides, respectively) and were comparable to that observed for unmodified LNA-TTP (-17.90 kcal/mol).[9a, 14] This analysis therefore suggests that the protecting groups do not interfere significantly with residues from the active site of the TdT polymerase.

In order to verify these favorable assets, we synthesized the suitably protected nucleoside triphosphate analogs starting from the corresponding 3'-*O*-protected nucleosides (Scheme 1). Application of the triphosphorylation method pioneered by Ludwig and Eckstein,[23] we obtained 3'-*O*-benzoyl-LNA-TTP **7** and 3'-*O*-pivaloyl-LNA-TTP **8** in moderate to good overall yields after anion exchange HPLC purification (see Methods and the Supporting Information).



Scheme 1. Synthesis of A) 3'-*O*-benzoyl-LNA-TTP **7** and B) 3'-*O*-pivaloyl-LNA-TTP **8**. Reagents and conditions: i) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; 2. $(n\text{Bu}_3\text{NH})_2$ $H_2P_2O_7$, DMF, $n\text{Bu}_3\text{N}$, rt, 45 min; 3. I_2, pyridine, H_2O, rt, 30 min, 30% over 3 steps for both **7** and 16% for **8**.

Template-independent primer extension (PEX) reactions

The TdT displays a remarkable tolerance for LNA and modified LNA nucleotides as substrates. However, irrespective of the chemical modification pattern present on the LNA

nucleotide, the TdT will incorporate only a single LNA unit at the 3' termini of DNA primers and synthesis is discontinued even if canonical nucleotides are fed to the reaction mixtures.[9a, b, 24] Hence, engineered versions of the TdT will likely be required to incorporate multiple, consecutive LNA nucleotides and hence permit controlled enzymatic synthesis of LNA-containing oligonucleotides. However, we deemed that TdT tailing experiments would give a first glimpse at the tolerance of polymerases for the modified nucleotides **7** and **8**. Therefore, we conducted template-independent primer extension (PEX) reactions with the TdT and the 19 nucleotide long, 5'-FAM-labelled primer **P1** (Supporting Information). Since the TdT has rather lax cofactor requirements,[20] we also evaluated the effect of three metal cations (i.e. Co^{2+}, Mn^{2+}, and Mg^{2+}) on the outcome of the TdT-mediated reactions. Modified nucleotide **7** appears to be a very good substrate for the TdT polymerase since n+1 product formation with complete conversion of the starting **P1** oligonucleotide could be observed (Figure 3). Indeed, complete conversion to the n+1 product can be observed at higher triphosphate concentrations (i.e. 1 mM) after one hour when Co^{2+} is used as a cofactor (Figure 3A). Under these experimental conditions, the incorporation efficiency of nucleotide **7** compares to that of unmodified LNA-TTP.[9a] While slightly longer reaction times (3h vs 1h) are required when Mn^{2+} is supplied to the reaction mixtures, also this cofactor allows for complete incorporation of the modified nucleotide **7** (Figure 3A). Moreover, Mg^{2+} appears as the least favorable cofactor even though complete conversion of the primer into the n+1 product could also be observed (Figure 3B). Lastly, when lower concentrations of triphosphates were used, the occurrence of faster running bands was observed. This probably is the result of hydrolytic degradation of the primer which has been reported for other modified nucleotides,[14, 25] Similarly, some weak bands with a gel mobility comprised between that of the n and n+1 products can be observed under specific conditions (Figure 3). These products might occur due to 3'-phosphorylation of primer **P1** by the TdT polymerase.[17b, 26] We have also analyzed the product stemming from a TdT-mediated incorporation reaction carried out in the presence of Co^{2+} and observed the formation of the expected n+1 product (m/*z* calcd: 6678.1781; found: 6678.1778; see Figure S9 Supporting Information). However, the presence of an oligonucleotide containing a single LNA-T nucleotide but without 3'-*O*-benzyl protecting group (m/*z* calcd: 6574.1519; found: 6574.1488; see Figure S9 in the Supporting Information) was also detected in this analysis. Such an ablation of the blocking group can either have occurred during the MS analysis itself or during the enzymatic reaction via an esterase activity of the TdT polymerase.

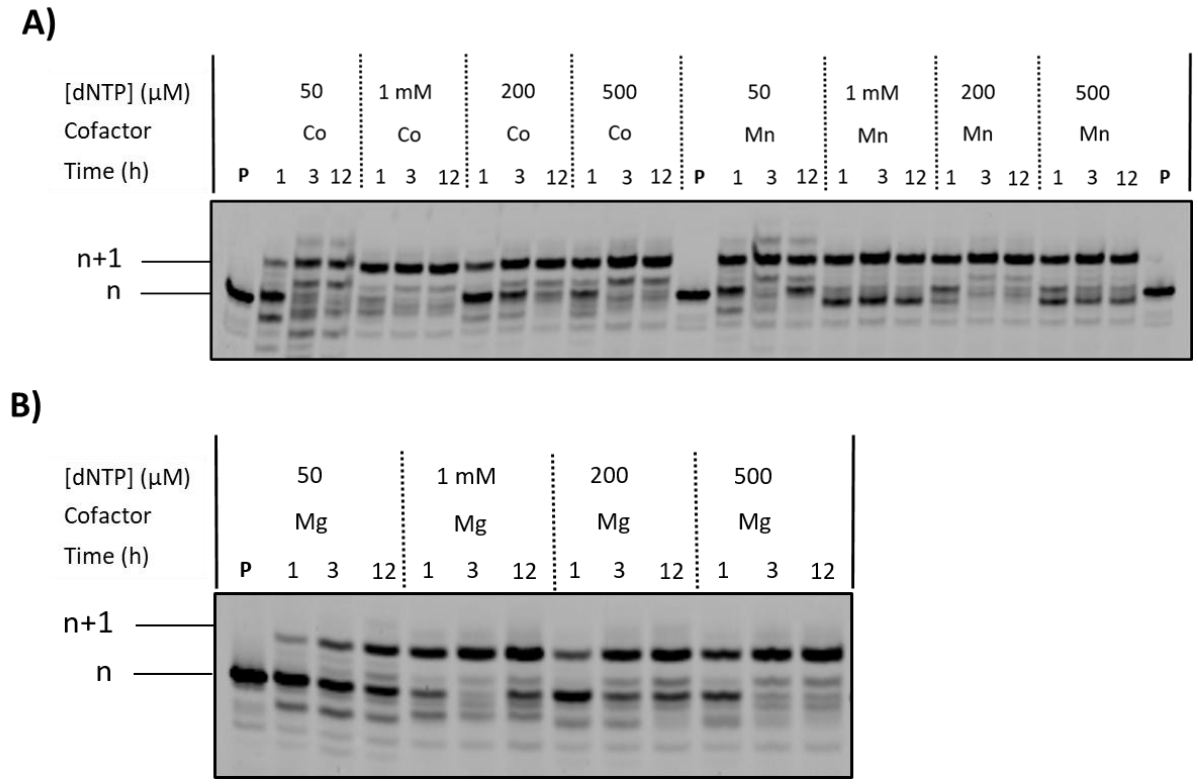


Figure 3. Gel images (PAGE 20%) of TdT-mediated tailing reactions with 3'-*O*-benzoyl-LNA-TTP **7** with A) 0.25 mM Co^{2+} and 1 mM Mn^{2+} and B) 1 mM Mg^{2+} as cofactors. Oligonucleotide **P1** (20 pmoles), TdT 10 U, and modified triphosphate at given concentrations were incubated with the corresponding cofactor at 37°C for different reaction times. **P** represents unreacted primer.

LNA nucleotide analog **8** equipped with a pivaloyl blocking group acted as a moderate substrate for TdT when assayed under similar PEX reaction conditions (Figure 4). Indeed, when Mn^{2+} was supplemented to the reaction mixtures, only 50% conversion to the n+1 product could be observed irrespective of other experimental conditions (i.e. reaction time and triphosphate concentration). A similar outcome was observed when Co^{2+} was used as metal cofactor, albeit more hydrolytic degradation of primer **P1** occurred simultaneously (Figure 4A). Lastly, only poor conversion (~20% yield) of primer to the expected n+1 product could be observed when Mg^{2+} served as cofactor for the enzyme (Figure 4B). The steric bulk of the pivaloyl moiety appears to be detrimental at least for TdT-mediated incorporation events.

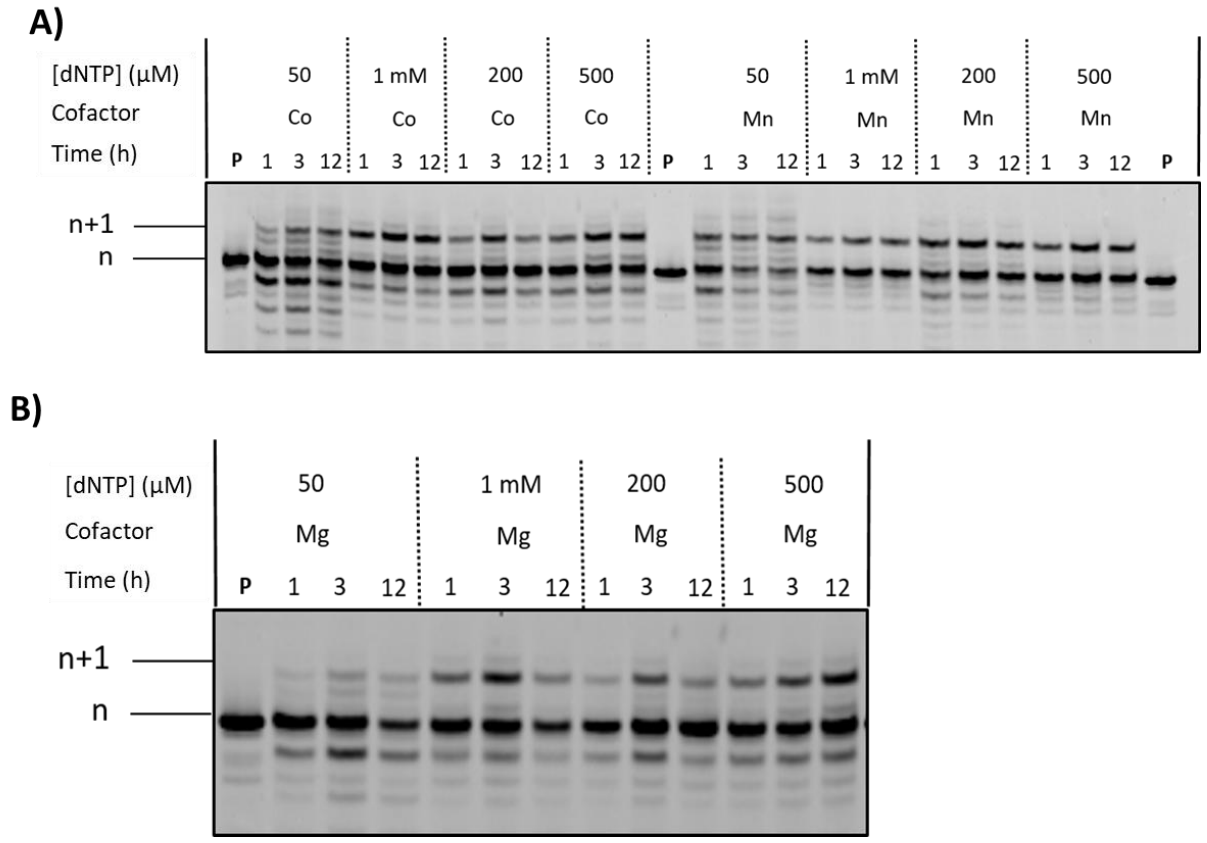
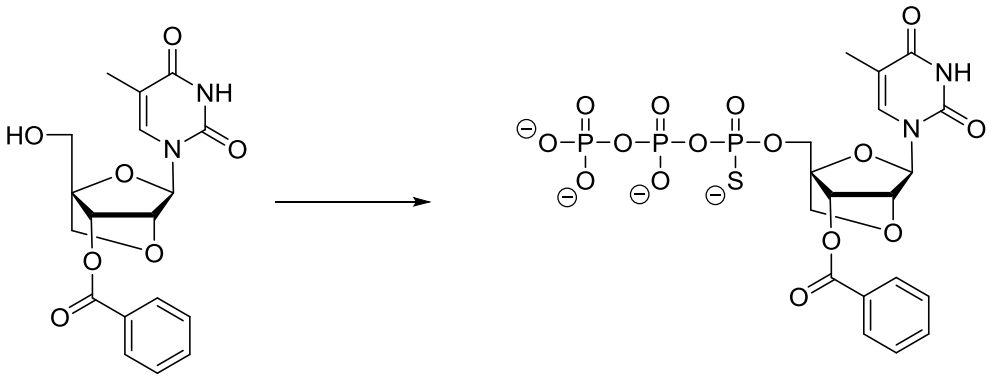


Figure 4. Gel images (PAGE 20%) of TdT-mediated tailing reactions with 3'-*O*-pivaloyl-LNA-TTP **8** with A) 0.25 mM Co^{2+} and 1 mM Mn^{2+} and B) 1 mM Mg^{2+} as cofactors. Oligonucleotide **P1** (20 pmoles), TdT 10 U, and modified triphosphate at given concentrations were incubated with the corresponding cofactor at 37°C for different reaction times. **P** represents unreacted primer.

Given the excellent substrate properties of nucleotide **7**, we wondered whether the presence of a 3'-*O*-benzoyl moiety could trigger the incorporation of a nucleotide containing an α-thiotriphosphate moiety which had been shown to be rather refractory to incorporation into ssDNA by the TdT polymerase.[9a] In order to verify this hypothesis, we synthesized 5'-α-thio-3'-*O*-benzoyl-LNA-TTP **9** by trapping the cyclic triphosphate moiety obtained by application of the Ludwig Eckstein protocol with the Beaucage reagent (Scheme 2).



Scheme 2. Synthesis of 5'-α-thio-3'-*O*-benzoyl-LNA-TTP **9**. Reagents and conditions: a) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; b) (*n*-$Bu_3NH)_2H_2P_2O_7$, DMF, *n*-Bu_3N, rt, 45 min; c) Beaucage reagent, pyridine, rt, 30 min, 10% over 3 steps.

With nucleotide **9** at hand, we carried out similar TdT-mediated PEX reactions as described above for nucleotide **7** (Figure 5). Even though the presence of the sulfur atom appears to impair the incorporation efficiency compared to either unmodified LNA-TTP or nucleotide **7**, conditions could be identified where the n+1 product formed in quantitative yields (Figure 5A and 5B). Concomitantly, nucleotide **9** appears to be a much better substrate for the TdT polymerase compared to 5'-α-thio-LNA-TTP where only moderate conversion yields (~50%) to the expected n+1 product could be achieved.[9a] We have also performed docking studies with both *R*p and *S*p configurations on the 5'-α-phosphate moiety of the incoming triphosphate (Figures S7 and S8, respectively). These simulations reveal that both diastereomers display less favorable binding energies than nucleotide **7** (-15.96 kcal/mol for *R*p and -15.46 kcal/mol for *S*p) but slightly improved, especially for the *R*p isomer, compared to unprotected 5'-α-thio-LNA-TTP.[9a] Also, the modified nucleotide **9** appears to be stabilized by the expected π-π stacking interaction with tryptophane 450, the negative charges of the triphosphate moiety seem to be stabilized by two positively charged residues (Lys338 and Arg336), and at least one conserved aspartic acid residue (Asp 345) is adequately positioned to harness a metal cofactor for catalysis as reported in structural analyses of the TdT in complex with canonical nucleotides.[27] Hence, addition of a 3'-*O*-benzoyl moiety appears to partially restore the substrate acceptance by the TdT polymerase on more demanding substrates such as LNA nucleotides bearing a 5'-α-phosphorothioate linkage. Overall, when assayed under template-independent DNA synthesis, nucleotides **7** and **9** acted as a good substrate and single extended products could be obtained efficiently in high yields. On the other hand, TdT-mediated polymerization reactions only proceeded sluggishly with nucleotide **8**, and n+1 product formation remained modest.

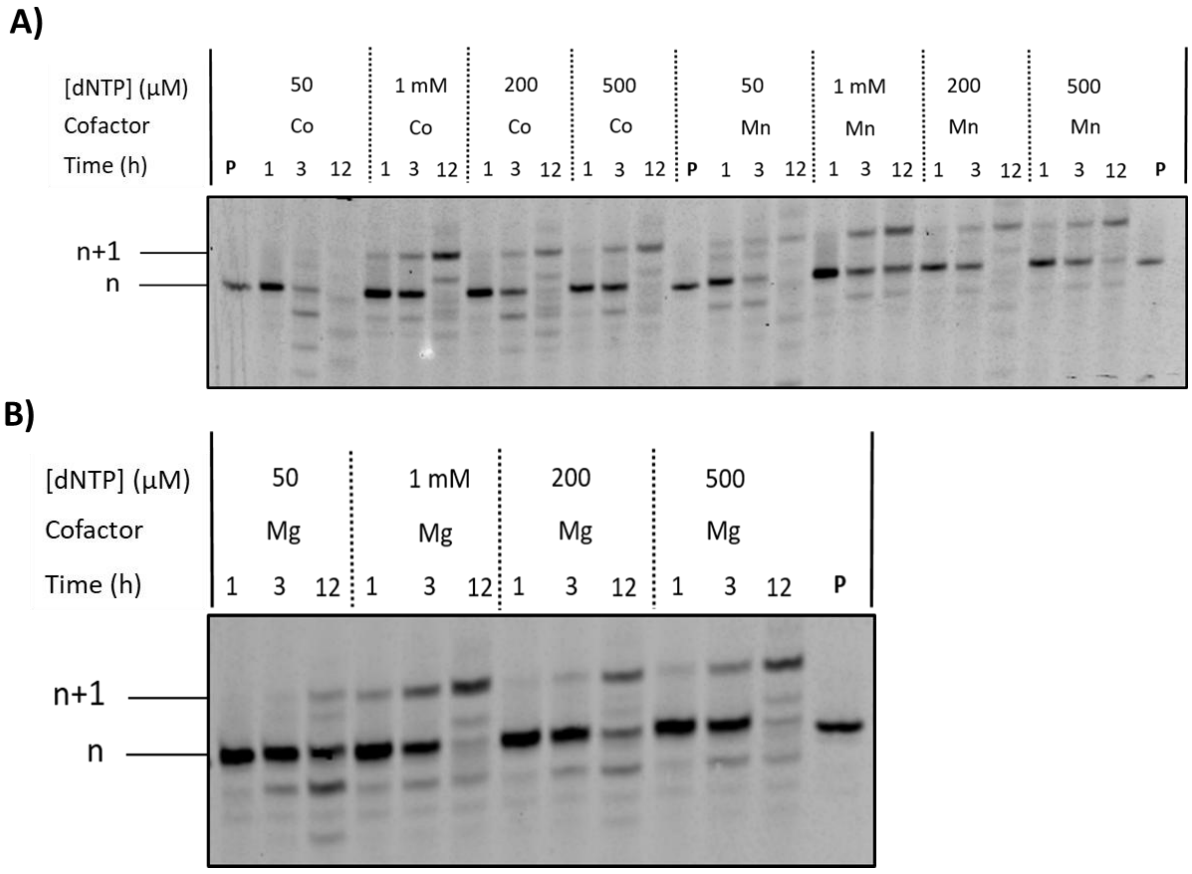


Figure 5. Gel images (PAGE 20%) of TdT-mediated tailing reactions with α-thio-3'-*O*-benzoyl-LNA-TTP **9** with A) 0.25 mM Co^{2+} and 1 mM Mn^{2+} and B) 1 mM Mg^{2+} as cofactors. Oligonucleotide **P1** (20 pmoles), TdT 10 U, and modified triphosphate at given concentrations were incubated with the corresponding cofactor at 37°C for different reaction times. **P** represents unreacted primer.

Template-dependent primer extension reactions

After demonstrating a degree of compatibility of the 3'-*O*-protected LNA nucleotides with TdT-mediated enzymatic reactions, we next sought to evaluate the possibility of using these analogs in template-dependent PEX reactions. Unlike TdT, A and B family DNA polymerases can incorporate multiple, even consecutive, LNA nucleotides and are likely to be more amenable to controlled enzymatic synthesis of therapeutic, LNA-containing oligonucleotides.[9c, 25a, 28] To this effect, we envisioned to use template **T1** that contains a five nucleotide long stretch of randomized nucleotides at the 3'-end (Supporting Information) and which can act as a universal template.[29] We carried out PEX reactions with nucleotides **7**-**9** and the **P2**/**T1** primer/template system (Figure S11). In this first screen, we considered various polymerases including Phusion which is known to accept LNA nucleotides as substrates.[28] In the case of 3'-*O*-benzoyl-LNA-TTP **7**, reactions conducted with HemoKlen Taq, *Bst*, Vent (*exo*$^-$) and the Klenow fragment of DNA polymerase I (Kf (*exo*$^-$)) led to the formation of the expected n+1 product albeit in moderate (~50%) yields (see Figure S11A). In some cases, for instance with *Bst* or Therminator, bands corresponding to an n+2 product

were also visible. This product could arise after removal of the protecting group due to the rather basic conditions employed during the enzymatic reactions, misincorporation of a natural nucleotide or an esterase activity of these polymerases (*vide infra*).[16] Similar results were obtained with 5'-α-thio-3'-*O*-benzoyl-LNA-TTP **9** except for a higher degree of formation of the n+2 product in most reactions (Figure S11B). Lastly, with 3'-*O*-pivaloyl-LNA-TTP **8** the best results were obtained with Kf (*exo*$^-$) as polymerase since primer **P2** was converted to the expected n+1 product in ~40% yield with little (~10%) n+2 product formation. All other reaction conditions led to either hydrolytic degradation of the primer or to an equal distribution of n+1 and n+2 products (Figure S11C). Based on this initial screen, it appears that some polymerases accept all these modified nucleotides as substrates without completely removing the blocking groups. Considering this rather positive feat, we next sought to optimize the experimental conditions using Vent (*exo*$^-$) and Kf (*exo*$^-$) as polymerases to promote the exclusive formation of the n+1 product with high yields. Starting with nucleotide **7**, we increased the concentration of triphosphate present in the reaction mixtures (Figure S12A), as well as the reaction times (Figure S12B). Increasing the amount of triphosphate present in the reaction mixture allowed to consume all primer with Vent (*exo*$^-$) but concomitantly produced higher fractions of the undesired n+2 product, while such an increase in concentration was highly detrimental to Kf (*exo*$^-$)-mediated reactions. On the other hand, the yield of n+1 product increased continuously with increasing reaction time when Kf (*exo*$^-$) served as polymerase while 3h long reaction catalysed by Vent (*exo*$^-$) led to complete conversion of the primer to the n+2 product. When the reaction time was further increased to 12h, 80% of the primer was converted mainly to the n+1 product by Kf (*exo*$^-$) (Figure S13). To verify the identity of both n+1 and n+2 products, we carried out an LCMS analysis of the reaction catalysed by Kf (*exo*$^-$) with a 12h long reaction time (Figure S10). The main peaks identified in the LCMS traces correspond to a primer with an added LNA-T nucleotide but devoid of 3'-*O*-benzoyl group (m/*z* calcd: 5540.971; found: 5540.977) as well as a primer with additional dG and LNA-T nucleotides (m/*z* calcd: 5870.023; found: 5870.026). Since a peak corresponding to primer **P2** missing a dG nucleotide was also observed, we hypothesize that the n+2 product arises via pyrophosphorolysis[30] of primer **P2** catalysed by the polymerase which results in a shorter primer and formation of dGTP, combined with incorporation of the resulting dGTP and one modified nucleotide **7** into DNA (the order of incorporation remains unclear). Thus, this LCMS analysis revealed that i) the n+2 product formation results from pyrophosphorolysis itself potentially stemming from the rather poor substrate acceptance of nucleotide **7** by Kf (*exo*$^-$) and ii) the 3'-*O*-benzoyl might be hydrolysed by the polymerase.

In order to suppress formation of the undesired n+2 product, we investigated whether the addition of DMSO (Figure S14A) or Mn^{2+} (Figure S14B) would improve the acceptance of nucleotide **7** by Kf (*exo*$^-$). Indeed, DMSO is often used to improve yields of enzymatic DNA synthesis due to its capacity at suppressing formation of secondary structures,[31] while mutagenic Mn^{2+} is a popular agent used to relax the specificity of polymerases.[32] However, addition of both agents revealed to be inefficient at reducing the formation of the n+2 product. Finally, we attempted to improve the ration of n+1 vs n+2 product formation by modulating simultaneously the reaction time and the concentration of nucleotide present in the reaction mixture (Figure 6). The best experimental conditions (i.e. 12h reaction time and 100 µM nucleotide) led to ~80% yield of n+1 product, ~15% n+2 product and ~5% of unreacted primer **P2**.

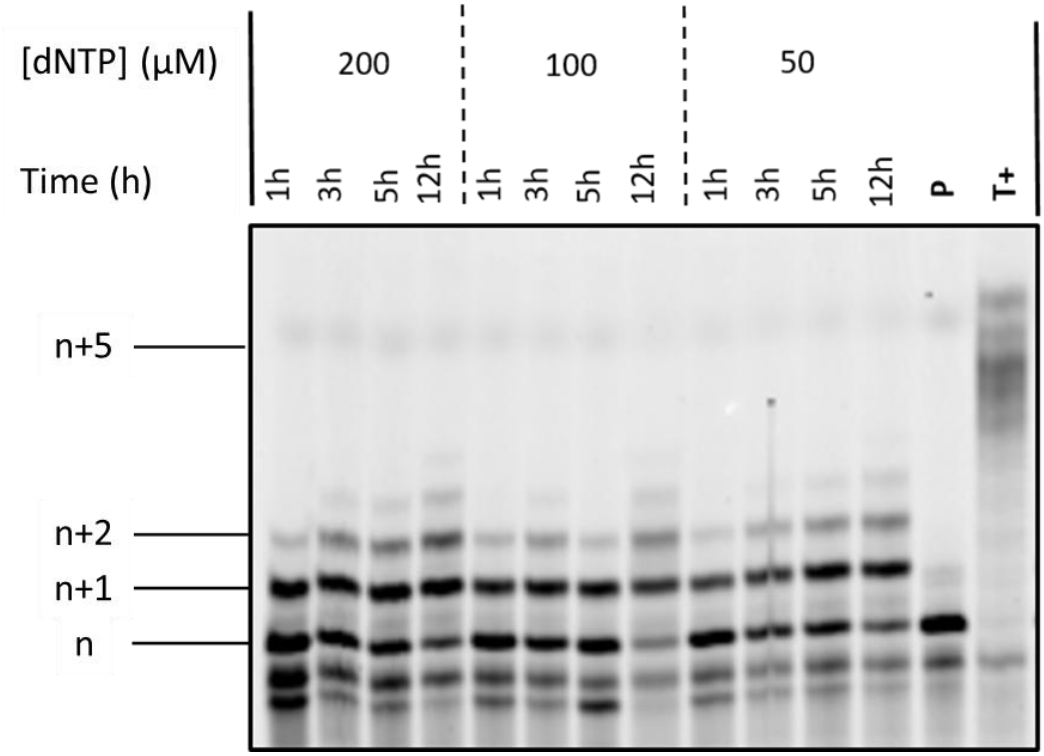


Figure 6. Gel (PAGE 20%) analysis of the effect of reaction time and nucleotide concentration on the outcome of PEX reactions performed with nucleotide **7** and primer **P2** and template **T1**. The reaction mixtures contained Kf (*exo*$^-$) (5 U) and were incubated at 37°C for given amounts of time. Positive control (**T+**): reaction carried out with all natural four nucleotides and Taq polymerase. **P** represents unreacted primer.

Next, we performed a similar analysis but with nucleotide analogs **8** and **9** and the best experimental conditions that were identified are highlighted in Figure 7. For 3'-*O*-pivaloyl-LNA-TTP **8**, near full consumption of primer **P2** and conversion to n+1 (~75%) and n+2 (~20%) products could be achieved under identical conditions as for nucleotide **7** (i.e. 12h reaction time and 100 μM nucleotide; Figure 7A). On the other hand, presence of the 5'-α-phosphorothioate moiety appears to suppress formation of the n+2 side-product albeit at the expense of slightly lower conversion yields of the primer (Figure 7B).

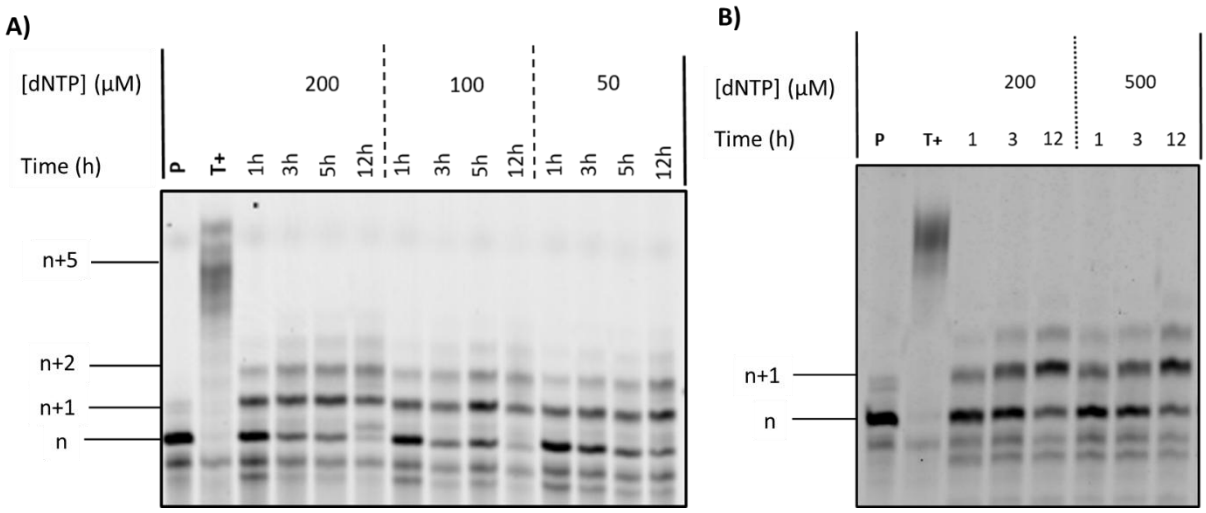


Figure 7. Gel (PAGE 20%) analysis of the effect of reaction time and nucleotide concentration on the outcome of PEX reactions performed with primer **P2** and template **T1** and A) nucleotide **8** and B) nucleotide **9**. The reaction mixtures contained Kf (*exo*$^-$) (5 U) and were incubated at 37°C for given amounts of time. Positive control (**T+**): reaction carried out with all natural four nucleotides and Taq polymerase. **P** represents unreacted primer.

Templates containing degenerate regions such as **T1** do not require the synthesis and storage of all possible combinations of sequences (i.e. 1024 distinct sequences with an N_5 region in **T1**) which is a clear advantage in the context of controlled enzymatic synthesis. On the other hand, the required templates (in our context a stretch of five dA nucleotide) are only minor products and hence the yields of enzymatic reactions might be impaired due to these low concentrations.[29] In order to verify whether the apparition of the n+2 side-product stems from a poor acceptance of nucleotides **7**-**9** by polymerases or rather arises from impeded enzymatic synthesis caused by the nature of the template, we have performed PEX reactions on a different primer/template system (Figure 8). To do so, we have opted for template **T2**[32b] which contains a single dA nucleotide located immediately after the 3'-end of the FAM-labelled primer **P1**. When nucleotides **7**-**9** were assayed under these conditions, no n+2 product formation could be observed and product n+1 formed in 100% after 12h for nucleotides **7** and **8**.

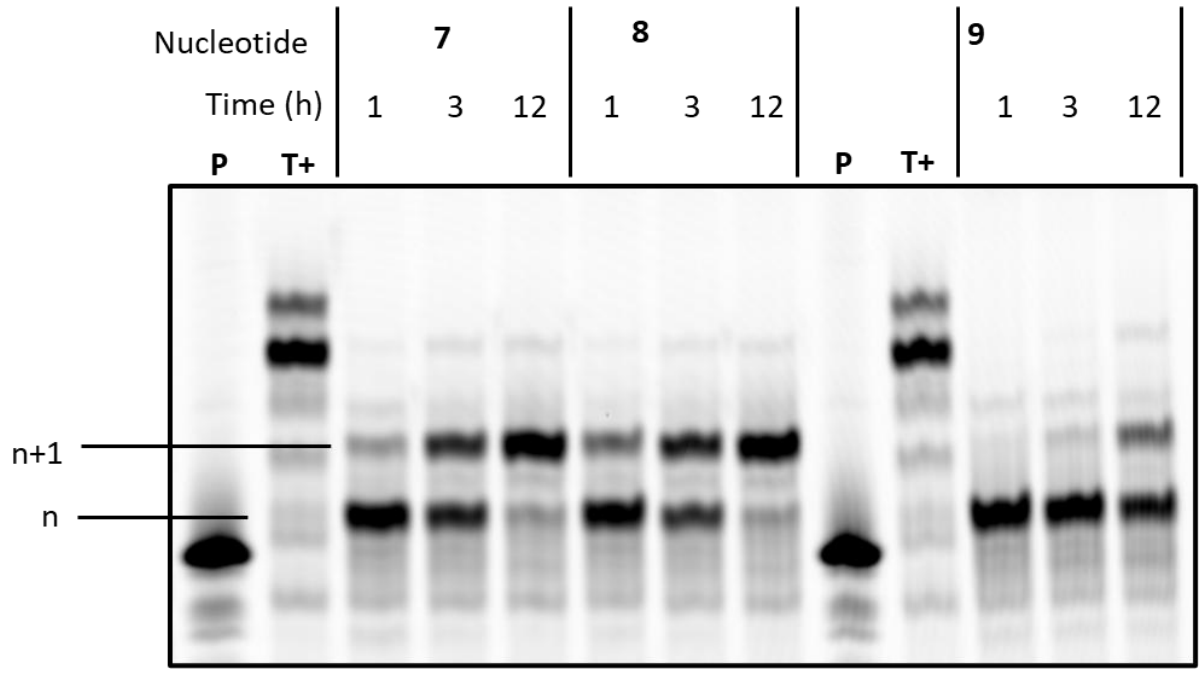
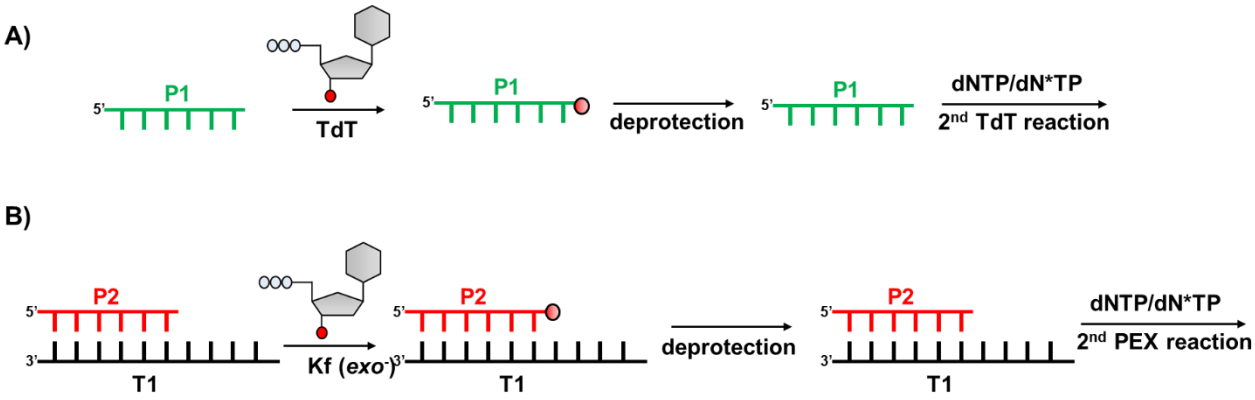


Figure 8. Gel (PAGE 20%) analysis of PEX reactions performed with primer **P1** and template **T2**. All reactions were performed in the presence of 100 µM of the modified triphosphates, Kf (*exo*$^-$) (5 U), at 37°C for 1h, 3h or 12h. Positive control (**T+**): reaction carried out with all natural four nucleotides and Taq polymerase. **P** represents unreacted primer.

After establishing that nucleotides **7** and **8** are compatible with template-dependent DNA synthesis but not with universal templates containing randomized regions, we sought to evaluate whether the protecting groups resisted the hydrolytic activity of polymerases. To do so, we installed single nucleotides **7** and **8** on primers **P1** and **P2** by TdT-mediated reactions (Scheme 3A) or by PEX reactions catalyzed by Kf (*exo*$^-$) (Scheme 3B), respectively. After subjecting the resulting products to a basic treatment followed by a purification by Nucleospin columns, a second enzymatic reaction is carried out with modified or natural nucleotides.



Scheme 3. Schematic representations of reactions to evaluate the hydrolytic removal of protecting groups by polymerases. A) Single nucleotides **7**, **8**, or LNA-TTP are installed on primer **P1**. The resulting products are then subjected to deprotection conditions (K_2CO_3 1M, 3h, at RT) and after purification, a second TdT reaction is initiated by the addition of fresh polymerase and either dTTP or modified nucleotides **7** or **8**; B) single nucleotides **7** or **8** are

installed by PEX reactions with Kf (*exo*$^-$) and primer **P2** and template **T1**. The resulting products are then subjected to a similar treatment as described in A).

While the second enzymatic reactions resulted in the addition of natural and modified nucleotides after the deprotection step, a similar outcome was observed in the absence of treatment K_2CO_3 (Figure 9). A similar trend was observed when primer **P1** and template **T2** were used in Kf (*exo*$^-$)-mediated PEX reactions (data not shown). Hence, these experiments suggest that the 3'-*O*-benzoyl- and 3'-*O*-pivaloyl-blocking groups are removed by the polymerase in the first step.

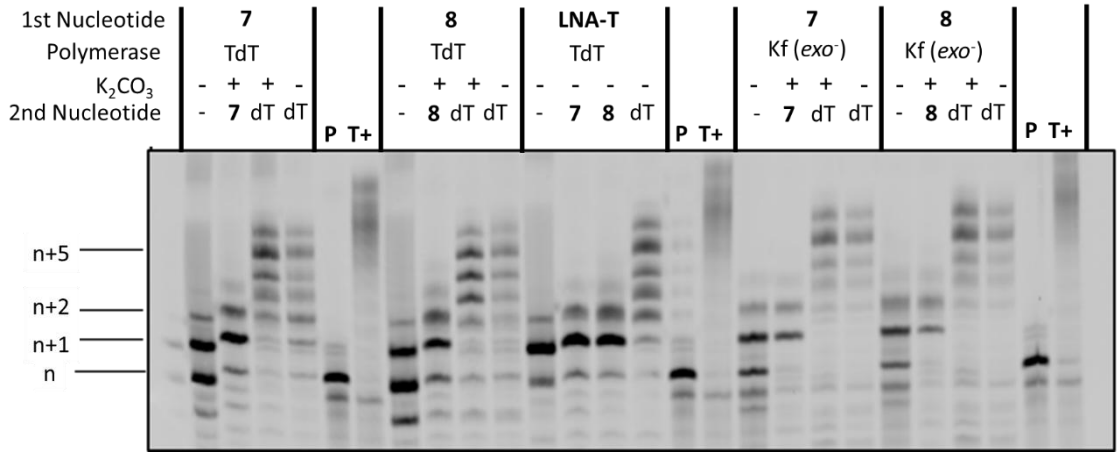


Figure 9. Gel (PAGE 20%) analysis of PEX reactions performed as described in Scheme 3. Modified triphosphates were at 100 µM and all reactions were carried out with TdT (10 U) or Kf (*exo*$^-$) (5 U) at 37°C for 12h. Positive control (**T+**): reaction carried out with all natural four nucleotides and Taq polymerase. **P** represents unreacted primer.

Conclusions

In controlled enzymatic synthesis, temporarily blocked nucleotides are incorporated by polymerases onto immobilized DNA sequences. Removal of the protecting group restores a free site for the introduction of another nucleotide in a subsequent synthetic cycle. This method can potentially lead to the synthesis of long (i.e. > 100 nucleotides) sequences with chemically modified nucleotides present at distinct positions. While some progress was made for standard DNA, particularly with 3'-*O*-NH_2[13b] and 3'-CH_2N_3[33] blocking groups, controlled enzymatic synthesis of XNAs is at a relative stage of infancy. Here, we have evaluated the possibility of using more stable protecting groups, including esters, ethers, and carbonates as reversible terminators for LNA synthesis. Of all explored protecting groups, benzoyl and pivaloyl appear to be stable upon incubation of the protected nucleosides in reaction buffer. The corresponding nucleotides are tolerated by the TdT polymerase even though longer reaction times are required for completion of the synthesis. When assayed under PEX reaction conditions on a universal template consisting of a stretch of randomized nucleotides, conversion to the expected n+1 product could be observed. Nonetheless, due to the nature of this template, an n+2 product stemming from a combination of pyrophosphorolysis and dual incorporation of modified and natural nucleotides was observed and could not be

suppressed. On the other hand, PEX reactions carried out with a different primer/template system did not lead to the formation of this side-product further demonstrating the compatibility of 3'-*O*-benzoyl- and 3'-*O*-pivaloyl-LNA-TTP with DNA polymerases under PEX reaction conditions. However, LCMS analysis revealed that polymerases were capable of removing these blocking groups via their rather efficient esterase activity. Taken together, this article represents an additional step in the understanding of the substrate tolerance of polymerases for XNA equipped with temporary blocking groups. While for LNA engineered polymerases might be more efficient for controlled enzymatic synthesis, this approach, as well as the identified 3'-O-pivaloyl- and 3'-O-benzoyl-blocking groups, might be more straightforward for other XNAs such as TNA[34] or FANA[1, 35] as well as for base-modified nucleotides particularly for those bearing epigenetic marks.[36] Future directions in this field will also involve development of solid-phase[37] and potentially micro-fluidic[38] methods to improve synthesis and facilitate automation combined with membrane technologies developed for peptide synthesis to wash off excess reagents.[39]

Experimental section

Materials and equipment:

All chemicals and solvents used were purchased from Sigma-Aldrich and Alfa Aesar unless stated otherwise. NMR spectra were recorded on a Bruker Avance 500 spectrometer (500.00 MHz for ^{1}H, 125.74 MHz for ^{13}C, and 202.51 MHz for ^{31}P) and all spectra were referenced to the signals of the corresponding solvent. Chemical shifts are given in ppm (δ scale) and coupling constants (*J*) in Hz. Assignation of the NMR signals was performed by using a combination of ^{1}H/^{1}H-COSY, ^{13}C-DEPT-135, and ^{13}C/^{1}H-HMBC experiments. High resolution electrospray ionization (ESI) mass spectra (MS, m/*z*) were recorded on a Waters Q-Tof Micro MS in the positive-ion electrospray ionization (ESI^{+}) mode. Solutions were prepared using 1:1 MeCN/H_2O containing 0.1% formic acid or MeOH/water containing 10 mM ammonium acetate in the case of sensitive compounds. HPLC purification was performed using an Äkta™ pure system (GE Healthcare) equipped with Thermo Scientific™ DNAPac™ PA100 preparative ion exchange column (13 µm, 250 x 22.0 mm). Unmodified DNA oligonucleotides were purchased from IDT. All the DNA polymerases (TdT, Phusion, Hemo KlenTaq, Taq, *Bst*, Q5, Therminator, Vent (*exo*$^{-}$), Dpo4, Deep Vent and Kf *exo*$^{-}$) were purchased from New England Biolabs as well as the natural dNTPs. Acrylamide/bisacrylamide (29:1, 40%) was obtained from Fisher Scientific. Visualization of PAGE gels was performed by fluorescence imaging using a using a Typhoon Trio phosphorimager with the ImageQuant software (both from GE Healthcare).

Synthesis of nucleotides:

Synthesis of 3'-*O*-benzoyl-LNA-TTP **7**

Nucleoside **1** was coevaporated twice with pyridine and dried under reduced pressure overnight before the reaction. Tributylammonium pyrophosphate was dried under reduced pressure overnight before the reaction.

Nucleoside **1** (75 mg, 0.200 mmol, 1 eq) was dissolved in dry pyridine (0.5 mL) and dried dioxane (0.6 mL) at room temperature under inert atmosphere. To this solution, 2-chloro-1,3,2-benzodioxaphosphorin-4-one (58 mg, 0.280 mmol, 1.4 eq) was added and the reaction mixture was stirred for 45 min. A solution of tributylammonium pyrophosphate (143 mg, 0.260 mmol, 1.3 eq), in dry DMF (0.5 mL) and tributylamine (0.3 mL) was added dropwise and the reaction mixture was stirred for another 45 min. It was then oxidized by the addition of iodine (81 mg, 0.320 mmol, 1.6 eq) in pyridine (1 mL) and H_2O (0.4 mL). After 30 min of stirring, the excess of iodine was quenched with a sodium thiosulfate solution (10% w/v in water) and the solution was concentrated under reduced pressure at 30°C. The residue was dissolved in H_2O and precipitated by the addition of $NaClO_4$ 2% in acetone. The crude product was purified by HPLC. Buffer A: 10 mM TEAB, Buffer B: 1mM TEAB. R_T = 19', 41%.

19 mg, 30% yield.

^{1}H NMR (500.02 MHz, D_2O): 7.94 (d, *J* = 8.00 Hz, 2H), 7.68 (s, 1H), 7.57 (t, *J* = 6.00 Hz, 1H), 7.41 (t, *J* = 8.00 Hz, 2H), 5.68 (s, 1H), 5.11 (s, 1H), 4.74 (s, 1H), 4.41-4.48 (m, 2H), 4.08-4.24 (m, 2H), 1.82 (s, 3H).

^{31}P NMR (202.51 MHz, D_2O): -10.55 (bs, 1P), -14.70 (d, *J* = 19.40 Hz, 1P), -25.75 (t, *J* = 19.60 Hz, 1P).

HRMS (ESI) for $C_{18}H_{20}N_2O_{16}P_3^-$ m/z calcd: 613.0031; found: 613.0031.

Synthesis of 3'-*O*-pivaloyl-LNA-TTP **8**

Nucleoside **4** was coevaporated twice with pyridine and dried under reduced pressure overnight before the reaction. Tributylammonium pyrophosphate was dried under reduced pressure overnight before the reaction. Nucleoside **4** (75 mg, 0.212 mmol, 1 eq) was dissolved in dry pyridine (0.5 mL) and dried dioxane (0.6 mL) at room temperature under inert atmosphere. To this solution, 2-chloro-1,3,2-benzodioxaphosphorin-4-one (61 mg, 0.296 mmol, 1.4 eq) was added and the reaction mixture was stirred for 45 min. A solution of tributylammonium pyrophosphate (150 mg, 0.276 mmol, 1.3 eq), in dry DMF (0.5 mL) and tributylamine (0.3 mL) was added dropwise and the reaction mixture was stirred for another 45 min. It was then oxidized by the addition of iodine (86 mg, 0.339 mmol, 1.6 eq) in pyridine (1 mL) and H_2O (0.4 mL). After 30 min of stirring, the excess of iodine was quenched with a sodium thiosulfate solution (10% w/v in water) and the solution was concentrated under reduced pressure at 30°C. The residue was dissolved in H_2O and precipitated by the addition of $NaClO_4$ 2% in acetone. The crude product was purified by HPLC. Buffer A: 10 mM TEAB, Buffer B: 1mM TEAB. R_T = 15', 35%.

20mg, 16% yield.

^{1}H NMR (400.13 MHz, D_2O): 7.60 (s, 1H), 5.60 (s, 1H), 4.81 (s, 1H), 4.57 (s, 1H), 4.33 (s, 2H), 3.98-4.06 (m, 2H), 1.78 (s, 3H), 1.06 (s, 9H).

^{31}P NMR (202.51 MHz, D_2O): -10.52 (bs, 1P), -14.70 (d, *J* = 19.20 Hz, 1P), -25.77 (t, *J* = 17.5 Hz, 1P).

HRMS (ESI) for $C_{16}H_{24}N_2O_{16}P_3^-$ m/z calcd: 593.0344; found: 593.0346.

Synthesis of α-thio-3'-*O*-benzoyl-LNA-TTP **9**

Nucleoside **1** was coevaporated twice with pyridine and dried under reduced pressure overnight before the reaction. Tributylammonium pyrophosphate was dried under reduced pressure overnight before the reaction. Nucleoside **1** (60 mg, 0.160 mmol, 1 eq) was dissolved in dry pyridine (0.4 mL) and dried dioxane (0.5 mL) at room temperature under inert atmosphere. To this solution, 2-chloro-1,3,2-benzodioxaphosphorin-4-one (46 mg, 0.224 mmol, 1.4 eq) was added and the reaction mixture was stirred for 45 min. A solution of tributylammonium pyrophosphate (114 mg, 0.208 mmol, 1.3 eq), in dry DMF (0.4 mL) and tributylamine (0.2 mL) was added dropwise and the reaction mixture was stirred for another 45 min. It was then oxidized by the addition of Beaucage reagent (51 mg, 0.256 mmol, 1.6 eq) in pyridine (0.5 mL). After 30 min of stirring, the solution was concentrated under reduced pressure at 30°C. The residue was dissolved in H_2O and precipitated by the addition of $NaClO_4$ 2% in acetone. The crude product was purified by HPLC. Buffer A: 10 mM TEAB, Buffer B: 1mM TEAB. R_T = 21', 56%.

10 mg, 10 % yield.

^{1}H NMR (500.02 MHz, D_2O): 7.94-7.96 (m, 2H), 7.71 (bs, 1H), 7.56-7.58 (m, 1H), 7.40-7.43 (m, 2H), 5.69 (bs, 1H), 5.13 (bs, 1H), 4.73 (bs, 1H), 4.44-4.56 (m, 2H), 4.06-4.24 (m, 2H), 1.85 (bs, 3H).

^{31}P NMR (202.51 MHz, D_2O): 40.68 (d, *J* = 28.20 Hz, 1P), 30.32 (d, *J* = 29.00 Hz, 1P), -14.07 (d, *J* = 17.80 Hz, 1P), -25.46 (t, *J* = 29.00 Hz, 1P), -27.28 (t, *J* = 22.60 Hz, 1P).

HRMS (ESI) for $C_{18}H_{20}N_2O_{15}P_3S^-$ m/z calcd: 628.9803; found: 628.9784.

General protocol of TdT-mediated extension reactions

Primer **P1** (20 pmol) is incubated with the modified nucleoside triphosphates (at a given concentration) with a metal cofactor (0.25 mM Co^{2+}, 1 mM Mn^{2+}, or 1 mM Mg^{2+}) and the TdT DNA polymerase (10 U) in 1X reaction buffer (supplied with the polymerase; 10 µL final volume) at 37 °C for indicated reaction times. The reaction mixtures were then purified by Nucleospin columns and quenched by the addition of an equal volume of loading buffer (formamide (70%), ethylenediaminetetraacetic acid (EDTA, 50 mm), bromophenol (0.1%), xylene cyanol (0.1%)). The reaction products were then resolved by electrophoresis (PAGE 20%) and visualized by phosphorimager analysis.

General procedure for PEX reactions

The template (15 pmol) was annealed to its complementary primer (10 pmol) by heating to 95 °C and slowly (over 30-45 min) cooling down to room temperature. The annealed oligonucleotides were then supplemented with modified and/or natural dNTPs (indicated concentrations) and polymerase (given concentrations in unit) in 1X reaction buffer. The reaction mixtures were then incubated at the recommended temperature for given amounts of time. The reaction mixtures were then purified by Nucleospin columns and quenched by the addition of an equal volume of loading buffer (formamide (70%), ethylenediaminetetraacetic acid (EDTA, 50 mm), bromophenol (0.1%), xylene cyanol (0.1%)).

The reaction products were then resolved by electrophoresis (PAGE 20%) and visualized by phosphorimager analysis.

Docking experiments

AutoDock version 4.2[21] was used for the docking simulation. The enzyme file was prepared using published coordinates (PDB 4I27). The magnesium atom was retained within the protein structure. A charge of +2 and a solvation value of -30 were manually assigned to the Mg atom. The molecules files were built on Biovia Discovery Studio® 4.5 and saved as pdb file. The docking area was assigned visually around the presumed active site. A grid of 40 Å x 40 Å x 40 Å with 0.497 Å spacing was calculated around the docking area using AutoGrid. We selected the Lamarckian genetic algorithm (LGA) for ligand conformational searching, which evaluates a population of possible docking solutions and propagates the most successful individual from each generation into the subsequent generation of possible solutions. For each compound, the docking parameters were as follows: trial of 20 dockings, population size of 150, random starting position and conformation, translation step ranges of 1.5 Å, rotation step ranges of 35°, elitism of 1, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06 and 2500000 energy evaluations. The docking method was first evaluated by redocking the corresponding ligand of the PDB structure and then docking of the molecules of interest in the TdT active site. The conformation of the obtained results was inspected and compared to the literature and crystal structures. The docking results from each of the compounds were clustered on the basis of the root-mean-square deviation (rmsd) of the Cartesian coordinates of the atoms and were ranked on the basis of free energy of binding. The top-ranked compounds were visually inspected for correct chemical geometry.

LCMS analysis of reaction products

Analysis of reaction products was carried out on a Thermo Vanquish UPLC system equipped with a binary pump, a column oven and DAD UV detector. A Waters Acquity Oligo BEH C18 50 x 2.1 mm, 1.7 µm, 130 Å column was used. We have used previously described gradients and methods.[14]

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Conflict of interest

The authors declare no conflict of interest.

Keywords

Controlled enzymatic synthesis; locked nucleic acids; modified nucleotides; polymerases; XNA.

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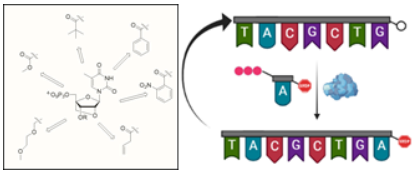

LNA nucleotides equipped with different 3'-*O*-blocking groups were evaluated for their compatibility with controlled enzymatic synthesis. Benzoyl- and pivaloyl-protected LNA nucleotides were readily accepted by polymerases.