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# Towards the controlled enzymatic synthesis of LNA containing oligonucleotides

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## Abstract

Enzymatic, *de novo* XNA synthesis represents an alternative method for the production of long oligonucleotides containing chemical modifications at distinct locations. While such an approach is currently developed for DNA, controlled enzymatic synthesis of XNA remains at a relative state of infancy. In order to protect the masking groups of 3'-*O*-modified LNA and DNA nucleotides against removal caused by phosphatase and esterase activities of polymerases, we report the synthesis and biochemical characterization of nucleotides equipped with ether and robust ester moieties. While the resulting ester-modified nucleotides appear to be poor substrates for polymerases, ether-blocked LNA and DNA nucleotides are readily incorporated into DNA. However, removal of the protecting groups and modest incorporation yields represent obstacles for LNA synthesis via this route. On the other hand, we have also shown that the template-independent RNA polymerase PUP represents a valid alternative to the TdT and we have also explored the possibility of using engineered DNA polymerases to increase substrate tolerance for such heavily modified nucleotide analogs.

## 33 1. Introduction

34 Synthetic oligonucleotides play essential roles in an increasing number of applications including  
35 storage of digital information in DNA,(Lee et al., 2019b;Doricchi et al., 2022b) drug  
36 discovery,(Lindenburg et al., 2020;Vummidi et al., 2022) and the development of mRNA  
37 vaccines.(Jackson et al., 2020;Chaudhary et al., 2021) Besides the need for production of larger  
38 numbers of sequences and scaling up to kilograms, demands vary widely in terms of size and also in  
39 sequence and chemical composition. For instance, antisense oligonucleotides consist of short, fully-  
40 modified sequences and the *de novo* genome synthesis requires the error-free assembly of massive  
41 amounts of shorter stretches of unmodified DNA.(Masaki et al., 2022;Matthey-Doret et al., 2022) On  
42 the other end of the spectrum, mRNA vaccines require the production of long (several thousands of  
43 nucleotides) oligonucleotides containing modified residues such as *N1*-methyl-pseudouridine(Nance  
44 and Meier, 2021;Dousis et al., 2022) while studies aiming at understanding the mechanisms and  
45 functions of larger RNAs such as long non-coding RNAs or mRNA call in for the synthesis of long,  
46 heavily modified sequences.(Zuckerman et al., 2020;Statello et al., 2021;Liu and Wang, 2022)

47 The main approach for the synthesis of oligonucleotides relies on the iterative addition of  
48 phosphoramidite-based building blocks on immobilized nucleic acid sequences.(Beaucage and  
49 Caruthers, 1981;Caruthers, 1985) While this method has met undeniable success, there are still  
50 inherently limiting factors. For instance, sequences longer than 200 nucleotides cannot be obtained  
51 by this solid-phase synthetic approach. In addition, the sustainability(Andrews et al., 2021) as well as  
52 the scalability(Molina and Sanghvi, 2019) of phosphorous(III)-based oligonucleotide synthesis are  
53 limited which negatively impacts scalable manufacturing.(Van Giesen et al., 2022) Hence, various  
54 enzymatic methods are currently developed to alleviate the shortcomings of solid-phase synthesis of  
55 nucleic acids. In this context, controlled enzymatic synthesis represents a promising approach where  
56 temporarily blocked nucleoside triphosphates are incorporated sequentially into DNA mainly by  
57 template-independent polymerases such as the terminal deoxynucleotidyl transferase (TdT).(Jensen  
58 and Davis, 2018;Lee et al., 2019b;Sarac and Hollenstein, 2019;Doricchi et al., 2022a;Lu et al.,  
59 2022;Van Giesen et al., 2022;Wang et al., 2022;Ashley et al., 2023;Hoose et al., 2023) The blocking  
60 groups can be affixed either at the 3'-hydroxyl moiety to prevent further nucleophilic attack on the  $\alpha$ -  
61 phosphorous of incoming nucleoside triphosphates(Bollum, 1962;Mackey and Gilham, 1971;Chen et  
62 al., 2010;Hutter et al., 2010;Gardner et al., 2012;Chen et al., 2013;Mathews et al., 2016;Jang et al.,  
63 2019) or on the nucleobase which then act as inhibitors of polymerases.(Bowers et al., 2009;Palluk et  
64 al., 2018) While robust protocols have been established for DNA, (Palluk et al., 2018;Lee et al.,  
65 2019b;Jung et al., 2022;Venter et al., 2022;Wang et al., 2022) changing the sugar chemistry to ribose  
66 (RNA) or to more complex modification patterns deviating from natural systems (xenonucleic acids,  
67 XNAs(Chaput and Herdewijn, 2019;Chaput et al., 2020)) raises yet unmet challenges. We have  
68 recently explored the possibility of using phosphate(Flamme et al., 2022a) or robust ester  
69 functionalities(Flamme et al., 2022b) as prosthetic 3'-*O*-protecting groups for controlled enzymatic  
70 synthesis of locked nucleic acids (LNAs). While some of these temporarily blocked XNA nucleotides  
71 are tolerated by various polymerases including TdT, intrinsic esterase(Canard et al., 1995;LinWu et  
72 al., 2019;LinWu et al., 2020) and phosphatase(Krayevsky et al., 2000;Flamme et al., 2022a) activities  
73 of polymerases precludes their use for the crafting of oligonucleotides. Here, we have explored i) the  
74 possibility of using yet more robust protecting groups designed to resist esterase and phosphatase  
75 activity for DNA and XNA synthesis with template-dependent and independent polymerases, ii)  
76 whether other template-independent polymerases than the TdT could be harnessed for *de novo* DNA  
77 and LNA synthesis, and iii) the use of engineered, template-dependent polymerases more tolerant to  
78 LNA nucleotides.

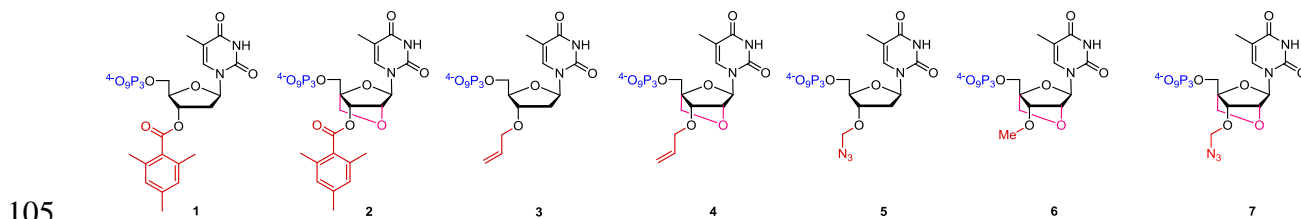
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## 80 2. Results and discussion

### 81 2.1 Design and synthesis of blocked DNA and LNA nucleotides

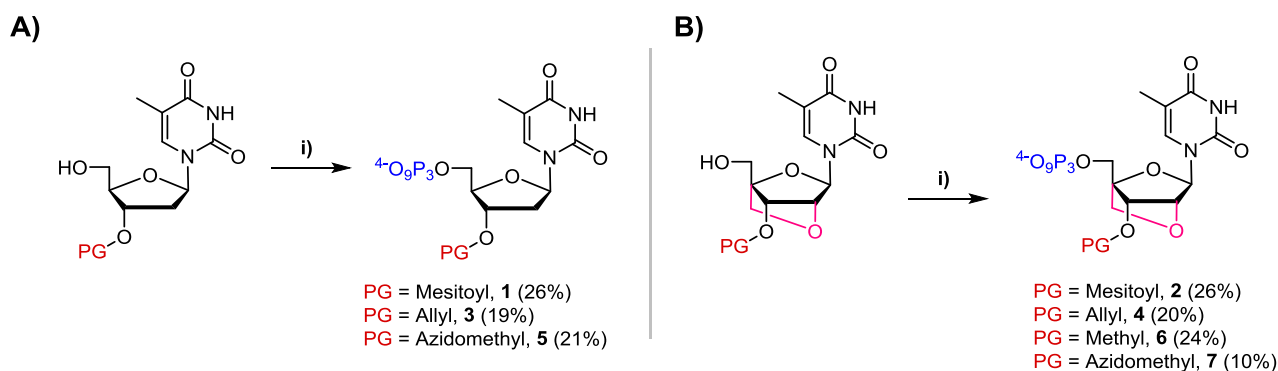
82 Benzoyl-protected LNA nucleotides were rather well-tolerated by a number of DNA polymerases  
83 and displayed an important resistance against hydrolytic removal.(Flamme et al., 2022b) Despite  
84 these favorable assets, some polymerases including *Kf(exo<sup>-</sup>)*, *Bst* or Terminator were capable of  
85 abstracting the benzoyl masking group by their moonlighting esterase activity leading to multiple  
86 incorporation events. Substitution of the aromatic moiety of benzoates with methyl groups not only  
87 decreases the rate of hydrolysis under mild acidic conditions compared to the unsubstituted parent  
88 compound but also to a change from an A-2 (Watson) mechanism involving a water molecule in the  
89 transition state to an A-1 (Ingold) mechanism that proceeds *via* the formation of an acylium  
90 ion.(Chmiel and Long, 1956;Shi et al., 2015;Pengthong et al., 2023) Based on this rationale, we  
91 deemed that the esterase activity of polymerases might be reduced by replacing a benzoyl- with a  
92 mesityl units on incoming nucleotides **1** and **2** (Figure 1).

93 Etherases catalyzing the hydrolysis of C-O bonds are quite rare in Nature and essentially hydrolyze  
94 aryl ether bonds in lignin(Picart et al., 2015), uncommon vinyl ethers,(Parsons et al., 2003) or lactyl  
95 ethers of MurNAc and related derivatives. This scarcity of naturally existing enzymes capable of  
96 hydrolyzing ether linkages is mainly due to the thermodynamic stability of C-O bonds.(Jaeger et al.,  
97 2005) This feature has already been exploited in nucleic acid chemistry to develop blocked  
98 nucleotides for sequencing purposes.(Ruparel et al., 2005;Ju et al., 2006;Wu et al., 2007;Guo et al.,  
99 2008;Keller et al., 2009;Knapp et al., 2011;Palla et al., 2014;Choi et al., 2022) Based on these  
100 considerations, we explored the possibility of using LNA nucleotides equipped with 3'-*O*-allyl  
101 (nucleotide **4**), 3'-*O*-methyl (nucleotide **6**), and 3'-*O*-azidomethyl (nucleotide **7**) protecting groups in  
102 controlled enzymatic XNA synthesis. In order to establish adequate control reactions, we also  
103 synthesized the known 3'-*O*-allyl- and 3'-*O*-azidomethyl-dTTP protected analogs (nucleotides **3**(Wu  
104 et al., 2007) and **5**(Guo et al., 2008), respectively).



106 **Figure 1.** Structures of designed DNA and LNA nucleotides **1-7** bearing 3'-*O*-blocking groups.

107 Based on this design, we first synthesized 3'-*O*-protected DNA and LNA nucleoside analogs starting  
108 either from 5'-*O*-DMTr- (for nucleotides **1-4** and **6**) or 5'-*O*-TBDMS-protected starting nucleosides  
109 (for nucleotides **5** and **7**) using protocols as described in detail in the supporting information and the  
110 literature.(K. Singh et al., 1998;Obika et al., 1998;Christensen et al., 2001) After installation of the  
111 3'-*O*-masking groups, the trityl and silyl protecting groups were removed under mild conditions.  
112 Finally, nucleoside triphosphates **1-7** were obtained in moderate yields (10-26%) by application of  
113 the one-pot-three-steps protocol developed by Ludwig and Eckstein(Ludwig and Eckstein, 1989)  
114 (Figure 2).



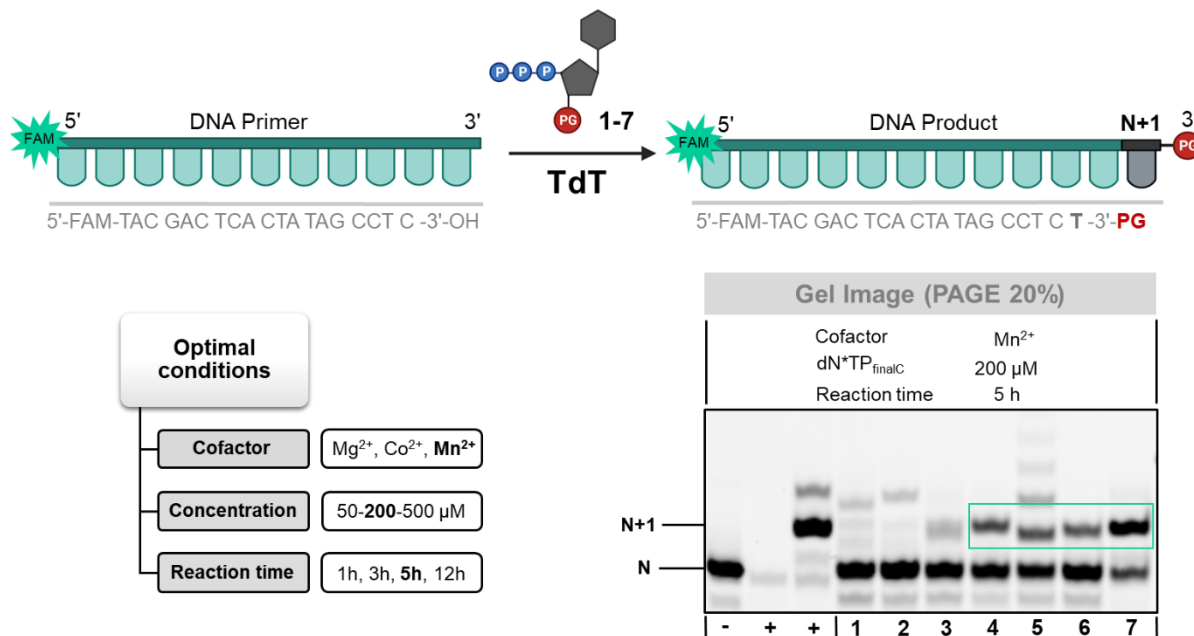
115

116 **Figure 2.** Synthesis of A) DNA and B) LNA nucleoside triphosphates **1-7** bearing 3'-*O*-blocking  
 117 groups. Reagents and conditions: **i)** 1) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine,  
 118 dioxane, rt, 1h; 2)  $(n\text{Bu}_3\text{NH})_2\text{H}_2\text{P}_2\text{O}_7$ , DMF,  $n\text{Bu}_3\text{N}$ , rt, 1h; 3)  $\text{I}_2$ , pyridine,  $\text{H}_2\text{O}$ , rt, 30 min.

## 119 2.2 Template independent synthesis

120 With blocked nucleotides **1-7** at hand, we sought to explore the possibility of constructing modified  
 121 and natural oligonucleotides using controlled enzymatic synthesis. In this context, template-  
 122 independent DNA polymerases such as the terminal deoxynucleotidyl transferase (TdT)(Sarac and  
 123 Hollenstein, 2019;Ashley et al., 2023) are often considered as prime candidates for *de novo* synthesis  
 124 of single-stranded DNA oligonucleotides.(Lee et al., 2019a;Jung et al., 2022;Lu et al., 2022;Wang et  
 125 al., 2022) While the TdT polymerase is rather tolerant to a broad array of structurally modified  
 126 nucleotides, it catalyzes the incorporation of single LNA nucleotides which then act as chain  
 127 terminators even in the absence of 3'-*O*-blocking groups.(Kuwahara et al., 2009;Kasahara et al.,  
 128 2010;Flamme et al., 2021) Nonetheless, extension reactions with the TdT and 3'-*O*-blocked LNA  
 129 nucleotides allow to rapidly gauge the substrate tolerance of a DNA polymerase for such modified  
 130 analogs. Therefore, we first evaluated whether nucleotides **1-7** could act as substrates for the TdT. To  
 131 do so, we incubated the modified nucleotides together with TdT, reaction buffer, various cofactors  
 132 ( $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Mg}^{2+}$ ), and a 19 nucleotide long, 5'-FAM-labelled DNA primer for various reaction  
 133 times (Figure 3 and Supplementary Figure 2). Azidomethyl-protected nucleotide **7** displayed the best  
 134 substrate tolerance by the TdT of all investigated nucleotides since conversion to the expected N+1  
 135 product could be achieved in near quantitative yields after 12h of reaction with  $\text{Mn}^{2+}$  as cofactor. In  
 136 addition to nucleotide **7**, 3'-*O*-allyl-blocked LNA analog **4** was also recognized as a substrate by the  
 137 TdT albeit with lower efficiency (~50% yield of conversion to N+1 product). Surprisingly, the  
 138 corresponding DNA counterparts **3** and **5** were not well recognized by the TdT and significant  
 139 amounts of further extended products could be detected by gel electrophoresis analysis. Analog **6**  
 140 equipped with a 3'-*O*-methyl group was incorporated into DNA by the TdT with moderate efficiency  
 141 (~30% of conversion), while nucleotides **1** and **2** were not recognized as substrates. It is noteworthy  
 142 mentioning that of all the conditions tested, the highest incorporation efficiencies were obtained,  
 143 irrespective of the nature of the modified nucleotide, when  $\text{Mn}^{2+}$  was used as cofactor along with 200  
 144  $\mu\text{M}$  triphosphate concentration and 5h or 12h of reaction time (Figure 3 and Supplementary Figure  
 145 2). This is in contrast with 3'-*O*-benzoyl and 3'-*O*-pivaloyl-protected LNA-TTPs which displayed a  
 146 marked preference for  $\text{Co}^{2+}$  over  $\text{Mn}^{2+}$ .(Flamme et al., 2022b) We then subjected the reaction  
 147 products obtained with nucleotides **4**, **6**, and **7** and the TdT to an LC-MS analysis (see Supplementary  
 148 Information for experimental details). When methylated LNA-TTP **6** was engaged in the reaction  
 149 mixture, the expected N+1 product could be detected by this analysis ( $m/z$  calcd.: 6588,1676;  
 150 observed: 6588,1960; see Supplementary Table 1). On the other hand, the 3'-allyl and 3'-  
 151 azidomethyl moieties of the detected N+1 products obtained with nucleotides **4** and **7** are clearly

152 absent. The main reaction products detected in these reactions was the N+1 product with 3'-OH  
 153 moiety ( $m/z$  calcd.: 6574,1519; observed: 6574,1834; see Supplementary Table 1). Collectively, these  
 154 results suggest the possibility that under longer reaction times and in the presence of  $Mn^{2+}$  cofactor,  
 155 ether protecting groups can be removed either as a consequence of the experimental conditions or  
 156 through the effect of the TdT polymerase.

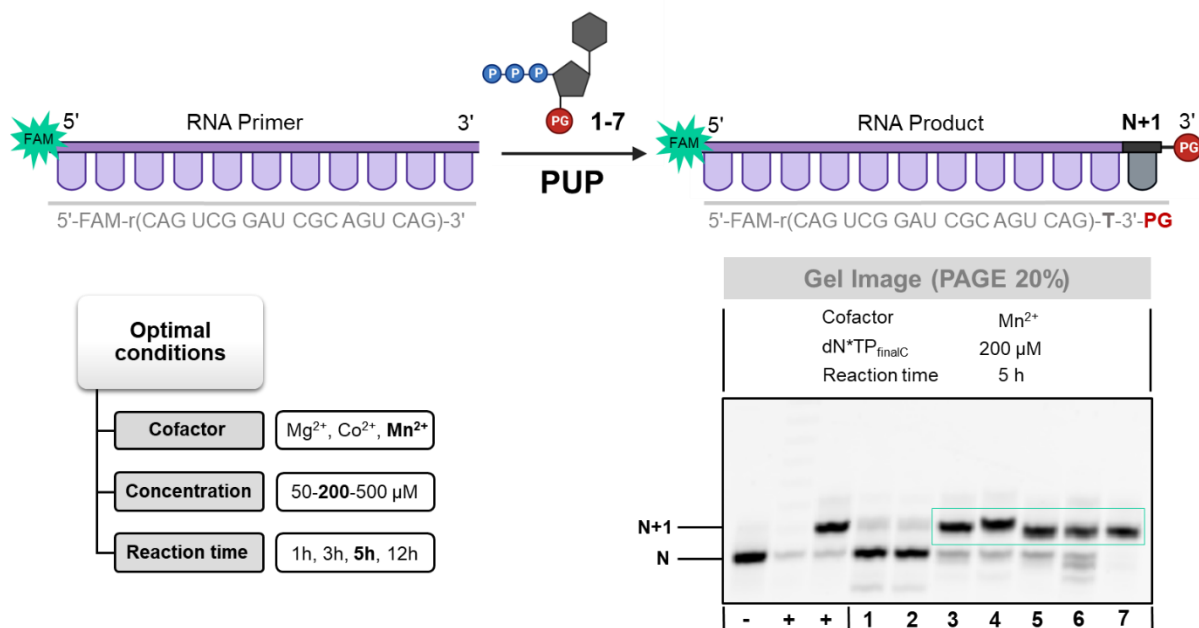


157

158 **Figure 3.** Gel image (PAGE 20%) of TdT-mediated tailing reactions with 3'-O-protected dN\*TPs **1-**  
 159 **7.** First from the left (+) – positive control using dTTP, second from the left (+) – positive control  
 160 using 3'-OH-LNA-dN\*TP, (-) – negative control in the absence of TdT.

161 Poly(U) polymerases (PUPs) are another class of template-independent polymerase that catalyze the  
 162 addition of rUMP residues at the 3'-termini of ssRNA in a mechanism reminiscent of that of the  
 163 TdT.(Kwak and Wickens, 2007;Munoz-Tello et al., 2012) PUPs have been employed for the terminal  
 164 labelling of RNA oligonucleotides and shown a relative tolerance for sugar- and base-modified  
 165 nucleotides.(Winz et al., 2012;George et al., 2020;Vo et al., 2021;Gupta et al., 2022) Surprisingly,  
 166 PUPs have not been considered for *de novo* synthesis of RNA or XNA oligonucleotides despite these  
 167 favorable assets. We thus evaluated the possibility of using PUPs to incorporate blocked and  
 168 unmodified LNA-TTP nucleotides into RNA given the structural preference of locked nucleic acids  
 169 for an A-type conformation.(Eichert et al., 2010;Campbell and Wengel, 2011a) To do so, we  
 170 incubated an 18 nucleotide long, 5'-FAM-labelled RNA primer with LNA-TTP and nucleotides **1-7**  
 171 with commercially available PUP under various experimental conditions including different  
 172 cofactors, reaction times, and nucleotide concentrations (Figure 4 and Supplementary Figure 3). With  
 173 the exception of nucleotides **1** and **2** equipped with 3'-O-mesitoyl groups, the RNA polymerase PUP  
 174 produced extended RNA primers with high efficiency (80-95% yields of conversion to N+1 product)  
 175 regardless of the nature of the nucleotide and the presence of blocking groups (see Figure 4). While  
 176 PUP incorporated a single, unblocked LNA-TTP with a similar efficiency as TdT on DNA  
 177 primers,(Kuwahara et al., 2009;Flamme et al., 2021) this RNA polymerase appears to be much more  
 178 tolerant to the presence of 3'-O-blocking groups than TdT. Indeed, DNA and LNA nucleotides  
 179 equipped with 3'-O-methyl-, 3'-O-allyl-, and 3'-O-azidomethyl- protecting groups were equally well  
 180 tolerated by PUP and successfully incorporated into RNA. Surprisingly, when the reaction product of

181 3'-OH-LNA-TTP was fed with UTP or increased concentrations of LNA-TTP no additional  
 182 incorporation events could be observed (data not shown). Hence, LNA acts as a chain terminator in  
 183 TdT- as well as in PUP-catalyzed reactions even in the absence of blocking groups. In addition, we  
 184 have analyzed the reaction products by LCMS to evaluate the nature of the products obtained by  
 185 PUP-mediated catalysis. To do so, we subjected the reaction products obtained with nucleotides **3-7**  
 186 and the PUP to an LCMS analysis (see Supplementary Information for experimental details). Unlike  
 187 what has been observed with the TdT, the expected N+1 products still equipped with their respective  
 188 protecting groups formed with nucleotides **3-6** (see Supplementary Table 2). On the other hand, the  
 189 product obtained with nucleotide **7** corresponds to the primer extended by a single LNA-T nucleotide  
 190 without any masking group at the 3'-end (*m/z* calcd.: 6636,9811; observed: 6637,0165; see  
 191 Supplementary Table 2). Clearly, reactions catalyzed by the PUP lead to the expected products with  
 192 little or no removal of the masking groups.



193

194 **Figure 4.** Gel image (PAGE 20%) of PUP-mediated tailing reactions with 3'-O-protected dN\*TPs **1-**  
 195 **7**. First from the left (+) – positive control using rUTP, second from the left (+) – positive control  
 196 using 3'-OH-LNA-dN\*TP, (-) – negative control in the absence of PUP.

### 197 2.3 Template dependent synthesis

198 While most efforts to improve the efficiency of *de novo* DNA synthesis are centered around TdT-  
 199 mediated, template independent oligonucleotide production, template-dependent approaches are also  
 200 emerging.(Hoff et al., 2020;Van Giesen et al., 2022;Hoose et al., 2023) A main advantage of  
 201 template-dependent synthesis is the plethora of polymerases that have been engineered to display  
 202 very lax substrate requirements and which might be capable of incorporating blocked nucleotides. On  
 203 the other hand, template-dependent synthesis leads to the formation of dsDNA *rather* than ssDNA  
 204 products but this can be circumvented by immobilizing products on solid-support or to an extent by  
 205 using universal templates.(Hoff et al., 2020;Flamme et al., 2022b) Consequently, we set out to  
 206 evaluate whether nucleotides **1-7** are compatible with enzymatic synthesis with template-dependent  
 207 polymerases. To do so, we performed primer extension (PEX) reactions using a 15-mer, 5'-FAM-  
 208 labelled primer and a 22-nucleotide long template equipped with a terminal poly(dA) stretch (Figure

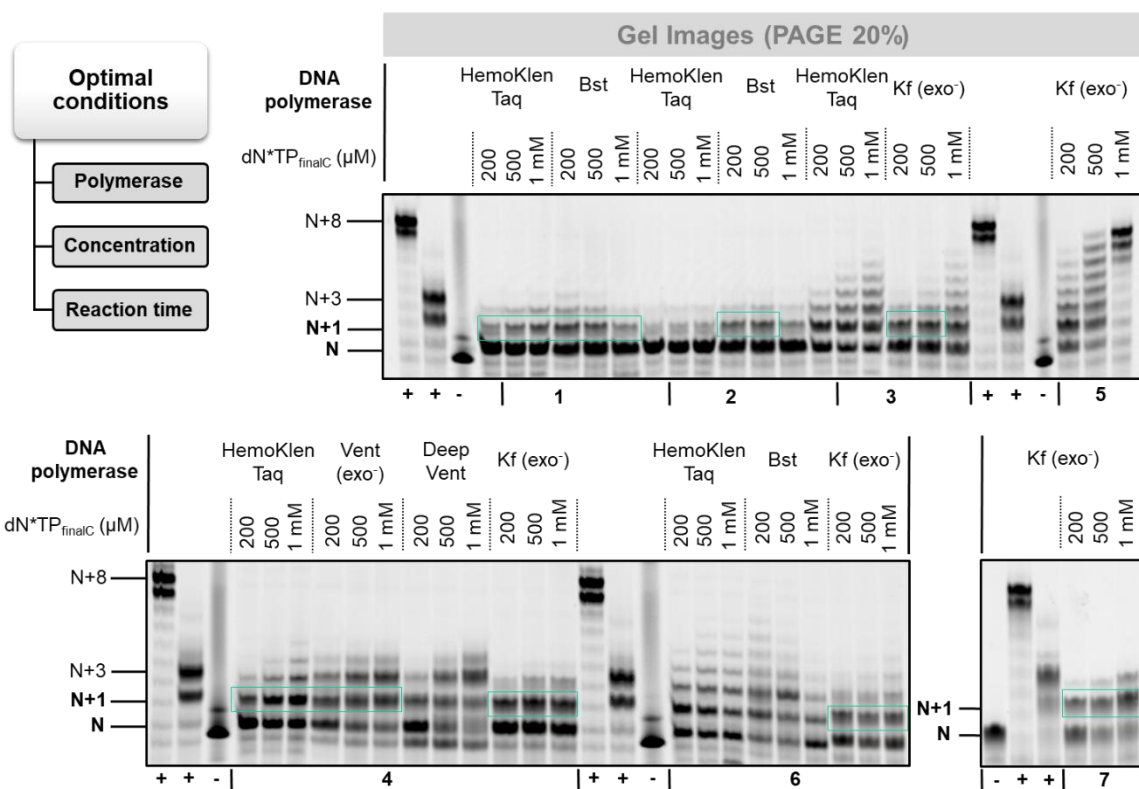
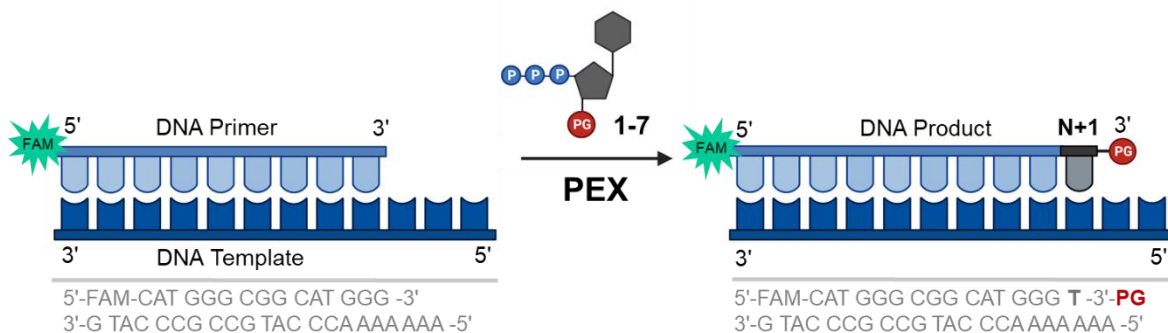
209 5). We then evaluated the capacity of a small subset of polymerases (spanning over three families (A,  
210 B, and Y): Hemo KlenTaq, *Bst*, Vent (*exo*<sup>-</sup>), *Sulfolobus* DNA polymerase IV, (Dpo4), Deep Vent,  
211 and Kf (*exo*<sup>-</sup>)) at accepting nucleotides **1-7** as substrates and extending the primer by one nucleotide  
212 (Figure 5 and Supplementary Figures 4a, 4b, and 4c). This analysis revealed that nucleotides **4** (3'-*O*-  
213 allyl-LNA-TTP), **6** (3'-*O*-methyl-LNA-TTP) and **7** (3'-*O*-azidomethyl-LNA-TTP) performed best of  
214 all evaluated analogs with over 50% of conversion of the primer to the expected N+1 product under  
215 optimized conditions. However, when higher dN\*TP concentrations were employed, N+2 product  
216 formation was observed suggesting partial removal of the blocking group (Supplementary Figures  
217 4a-c). On the other hand, increasing the reaction time to 12h led to a near completion of the primer  
218 and exclusive formation of the N+1 product (Supplementary Figure 4d). Nucleotides **3** and **4**  
219 equipped with 3'-*O*-allyl groups were tolerated by polymerases such as Kf (*exo*<sup>-</sup>) but led to lower  
220 conversion yields (30-40% of N+1 product formation). Interestingly, nucleotides **1** and **2** equipped  
221 with bulky ester groups were incorporated to a certain extent by the Taq and *Bst* polymerases under  
222 PEX reaction conditions unlike what had been observed with both the TdT and the PUP polymerases.  
223 Even though yields remained modest (~20%), these incorporation events highlight the difference in  
224 substrate tolerance at the level of position 3' of the deoxyribose sugar between template-dependent  
225 and template-independent polymerases. Lastly, reactions carried out with DNA nucleotide **5** (3'-*O*-  
226 azidomethyl-dTTP) led to the formation of a product distribution unlike those performed with the  
227 corresponding LNA analog. Overall, incorporation efficiencies in PEX reactions were comparable to  
228 those observed for related nucleotides blocked with ester moieties (Flamme et al., 2022b) and  
229 nucleotide **7** appeared to be the most promising candidate.

230 As for reactions catalyzed by template-independent polymerases, we subjected the resulting PEX  
231 reaction products to a thorough LCMS analysis to i) verify whether expected products were formed  
232 and ii) shed light into the product distribution observed with nucleotide **5** as well as the nature of the  
233 N+2 products. Analysis of the reaction products of nucleotides **3** and **4** obtained with Kf (*exo*<sup>-</sup>)  
234 clearly highlights formation of the expected N+1 *but* without the 3'-*O*-allyl groups in both cases  
235 (Supplementary Table 3). While the removal of an allyl ether groups was not expected, these results  
236 are similar to those obtained with the TdT polymerase with these blocked nucleotides. Unfortunately,  
237 no product other than phosphorylated template could be observed in the reaction mixtures with  
238 nucleotides **1**, **2**, and **6**. Intrigued by these results, we tried to rationalize the loss of protecting groups  
239 observed by gel electrophoresis and LCMS analysis. Concerning the azidomethyl protecting group,  
240 we believed this to arise due to the presence of the reducing agent dithiothreitol (DTT) both in the  
241 reaction and storage buffers of polymerases. We thus performed a PEX reaction with nucleotide **7**  
242 and Kf (*exo*<sup>-</sup>) purchased without DTT in the storage buffer and in a reaction mixture devoid of the  
243 reducing agent (Supplementary Figure 4e). However, gel electrophoresis of the reaction product  
244 obtained after 12h also showed the formation of the N+2 product. Addition of dTTP to the reaction  
245 mixture led to the formation of a product distribution thus suggesting partial removal of the  
246 protecting group. Lastly, treatment of the reaction mixture with potassium carbonate (1M, 3 hours,  
247 RT) followed by incubation with canonical dTTP led to the same outcome. Overall, LCMS analysis  
248 combined with additional PEX reactions revealed that ether-blocked nucleotides were incorporated  
249 into DNA by polymerases but that the blocking groups were abstracted during the reactions.

250 In order to improve the yield of N+1 product formation, we considered generating a mutant of the  
251 KOD polymerase that would tolerate LNA nucleotides additionally modified at position 3' of the  
252 sugar moiety. We thus considered engineering a KOD polymerase variant that contained one point  
253 mutation at the level of the exonuclease domain (P179S) and one in the thumb section (L650R).  
254 Point mutations were introduced at these sites because these have been recognized as facilitating the  
255 incorporation of sugar-modified nucleotides (Bergen et al., 2013; Larsen et al., 2016; Hoshino et al.,



256 2020;Hajjar et al., 2022) and KOD was chosen given its tolerance for LNA nucleotides.(Veedu et al.,  
257 2010) With this KOD mutant HP1.C2 at hand (courtesy from Roche), we carried out PEX reactions  
258 with blocked nucleotides **1-7** as well as unblocked LNA-TTP using similar conditions as described  
259 above. First, we carried out PEX reactions with LNA-TTP and canonical dTTP to evaluate the  
260 proficiency of KOD mutant HP1.C2. Gel electrophoretic analysis revealed that the polymerase was  
261 capable of fully extending the primer with dTTP resulting in an N+8 product (which corresponds to  
262 full length product with an additional, untemplated addition). On the other hand, LNA-TTP was  
263 accepted as a substrate however only three nucleotides were incorporated into the primer  
264 (Supplementary Figure 4c). Similar efficiencies have been observed with other DNA  
265 polymerases.(Flamme et al., 2021) We then extended our study to blocked nucleotides and gel  
266 electrophoretic analysis of the reaction products revealed that nucleotides **1, 2, 3, and 6** were not  
267 tolerated at all by this polymerase since no or very little (<10% conversion of the primer) extended  
268 product could be observed (Supplementary Figures 4a and 4c). On the other hand, LNA nucleotide **4**  
269 was readily incorporated into DNA by the mutant polymerase but a lower running band suggested  
270 that undesired N+2 product formed while a faster running band suggested some partial hydrolytic  
271 degradation of the primer. Similarly, PEX reaction with DNA nucleotide **5** was readily incorporated  
272 and led to a distribution of N+1 and N+2 but no hydrolytic degradation of the primer. As noticed for  
273 other polymerases, nucleotide **7** acted as an excellent substrate for KOD mutant HP1.C2, since  
274 primer was fully converted (Supplementary Figure 4c). However, the main product stemming from  
275 this reaction was that corresponding to a double incorporation event (N+2) suggesting an abstraction  
276 of the protecting group during the reaction.



277

278 **Figure 5.** Gel image (PAGE 20%) of PEX reactions with 3'-O-protected dN\*TPs **1-7**. First from the  
 279 left (+) – positive control using dTTP, second from the left (+) – positive control using 3'-OH-LNA-  
 280 dN\*TP, (-) – negative control in the absence of polymerase.

### 281 3. Discussion

282 Chemical synthesis of XNAs is particularly efficient for the development of potent therapeutic  
 283 oligonucleotides (mainly antisense and siRNA)(McKenzie et al., 2021) but is more challenging for  
 284 longer (> 50 nucleotide-long) sequences.(Taylor et al., 2016) On the other hand, polymerase-  
 285 mediated synthesis grants access to very long sequences(Hoshino et al., 2020) but control of the  
 286 localization of the modified nucleotides within the sequence is limited. The combination of both  
 287 methods appears to be a potential strategy for the preparation of long oligonucleotides with  
 288 modifications present at user defined positions. However, to reach these aims polymerases need to  
 289 circumvent multiple hurdles in XNA *de novo* synthesis. Indeed, polymerases need to cope with  
 290 modifications present at both the level of the sugar and the 3'-position. In addition, the masking  
 291 group needs to be stable for longer time storage but concomitantly should be removable under mild  
 292 conditions that would not affect the integrity of DNA and XNA oligonucleotides. Lastly, both

293 incorporation of the blocked nucleotides and removal of the masking groups need to be high yielding  
294 and fast to be considered for the synthesis of longer oligonucleotides. In order to unravel such a  
295 potential protecting group candidate, we have focused on the *de novo* synthesis of LNA-containing  
296 oligonucleotides due to the relevance of this type of chemical modification in the context of  
297 therapeutic oligonucleotides.(Campbell and Wengel, 2011b;Hagedorn et al., 2018) In addition, LNA-  
298 TTP appears to be a more difficult substrate for polymerases since the presence of such a  
299 modification often induces rather high error-rates.(Pinheiro et al., 2012;Hoshino et al., 2020) So far,  
300 we have synthesized LNA nucleoside triphosphates equipped with a variety of 3'-*O*-blocking groups  
301 including phosphate, esters, and ethers. Nucleotides equipped with 3'-*O*-phosphate units suffer from  
302 poor polymerase acceptance due to the increased negative charge and relative bulkiness of the  
303 modification but also from rapid removal by the inherent phosphatase activity of  
304 polymerases.(Flamme et al., 2022a) Ester protecting groups are better tolerated by polymerases but a  
305 fine balance between removal by the esterase capacity of polymerases and efficient incorporation  
306 needs to be evaluated in a case to case manner.(Flamme et al., 2022b) Here, we have extended this  
307 approach to ether protecting groups and we have found that these nucleotides could be incorporated  
308 into DNA by template-independent and template-dependent reactions using DNA polymerases.  
309 However, incorporation efficiencies for N+1 product formation rarely exceed 60-70% which is  
310 clearly unpractical for *de novo* XNA synthesis. In addition, removal of most of the 3'-*O*-ether  
311 protecting groups was observed and clearly results from an abstraction event during enzymatic  
312 reactions. The reasons for these unexpected ablations of the protecting groups remain unknown and  
313 might include reduction by DTT or other reagents, combined presence of divalent metal cations and  
314 long reaction times, or an etherase activity of polymerases. Interestingly, the RNA polymerase PUP  
315 incorporates only single LNA nucleotides which then act as chain terminators but unlike the TdT  
316 which displays a similar behaviour, the PUP is much more tolerant to the presence of 3'-  
317 modifications. Understanding the reason why LNA nucleotides act as chain terminators for reactions  
318 catalyzed by the TdT and the PUP polymerases would allow to engineer mutant enzymes that would  
319 be ideal candidates for *de novo* LNA and potentially XNA synthesis.

#### 320 4. Conclusions

321 Here, we report the synthesis of various DNA and LNA nucleotides blocked with 3'-*O*-ether and  
322 more robust 3'-*O*-ester protecting groups, and their further biochemical evaluation in enzymatic  
323 reactions. We have shown that nucleotides equipped with ether linkages were tolerated by DNA and  
324 RNA polymerases while the ester moieties precluded incorporation into oligonucleotides presumably  
325 due to the increased bulkiness of the blocking group. We have also shown that the PUP polymerase  
326 readily tolerates 3'-*O*-masked LNA nucleotides as substrates and thus represents a valid alternative  
327 polymerase to be considered for *de novo* synthesis of XNA oligonucleotides. Similarly, we have  
328 evaluated the possibility of using an engineered DNA polymerase to increase product formation.  
329 Surprisingly, LCMS and gel electrophoresis analysis revealed that most ether linkages were  
330 abstracted during the enzymatic reactions. Hence, future directions for improving XNA *de novo*  
331 synthesis will include the evaluation of other protecting groups, engineered versions of the TdT and  
332 PUP polymerases, and potentially considering polymerases with the capacity to catalyze the  
333 formation of other linkages such as phosphoramidate bonds.(Aggarwal et al., 2022)

334

### 335 **Data availability statement**

336 The original contributions presented in the study are included in the article/Supplementary Material,  
337 further inquiries can be directed to the corresponding author.

### 338 **Author contributions**

339 N.S., D.K., K.P., M.F., and M.K. designed the synthetic pathways and performed synthesis of all  
340 compounds, N.S. and M.F. carried out all enzymatic extension reactions, A.S. carried out all the LC-  
341 MS analyses of enzymatic reaction products, N.S., M.F., A.S., S.H., S.B., K.P., and M.H. analyzed  
342 the data, M.H. designed and wrote the manuscript, N.S., S.H., K.P., and M.H. designed the project,  
343 S.H., K.P., and M.H. acquired funding and administered the project, and all authors proof-read and  
344 edited the manuscript.

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### 350 **Conflict of interest**

351 Authors A.S., S.H., S.B., K.P., and F.S. are employed by F. Hoffmann-La Roche Ltd. This study  
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