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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 α -
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.

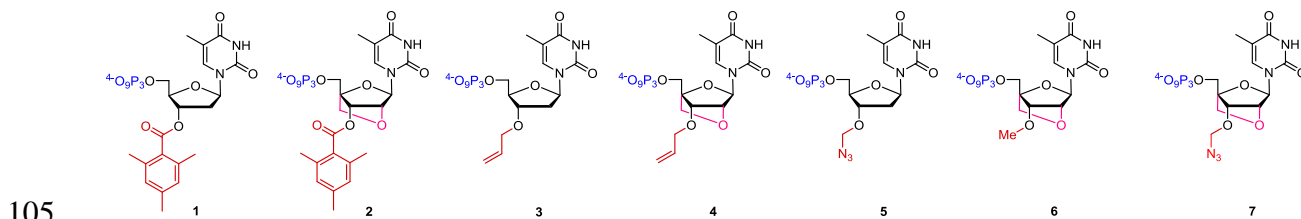
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80 2. Results and discussion

81 2.1 Design and synthesis of blocked DNA and LNA nucleotides

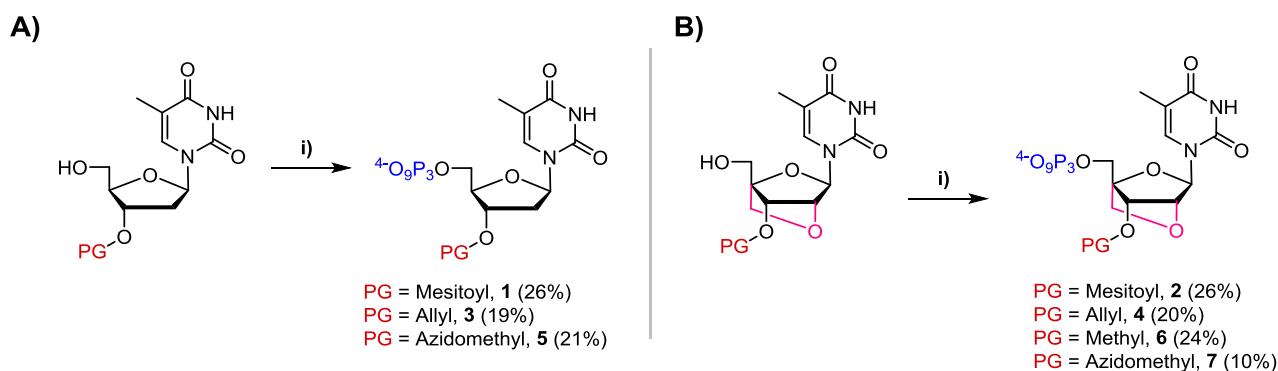
82 Benzoyl-protected LNA nucleotides were rather well-tolerated by a number of DNA polymerases
83 and displayed an important resistance against hydrolytic removal.(Flamme et al., 2022b) Despite
84 these favorable assets, some polymerases including *Kf(exo⁻)*, *Bst* or Terminator were capable of
85 abstracting 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).



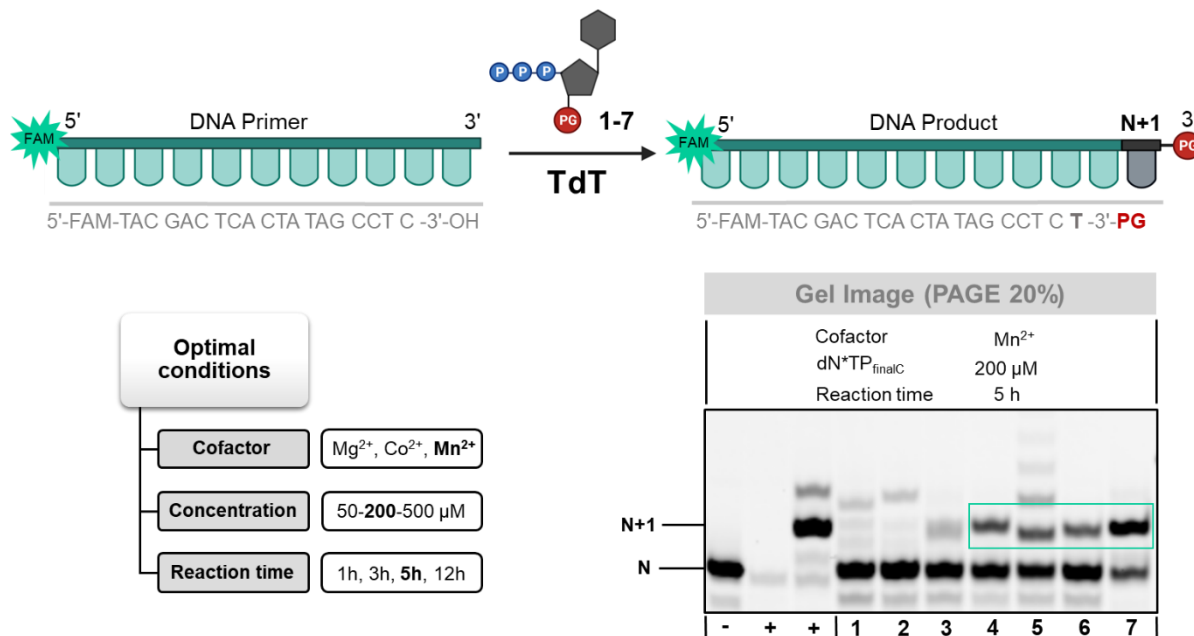
115

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) I_2 , pyridine, H_2O , 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 (Co^{2+} , Mn^{2+} , or 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 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 Mn^{2+} was used as cofactor along with 200
 144 μM 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 Co^{2+} over 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.

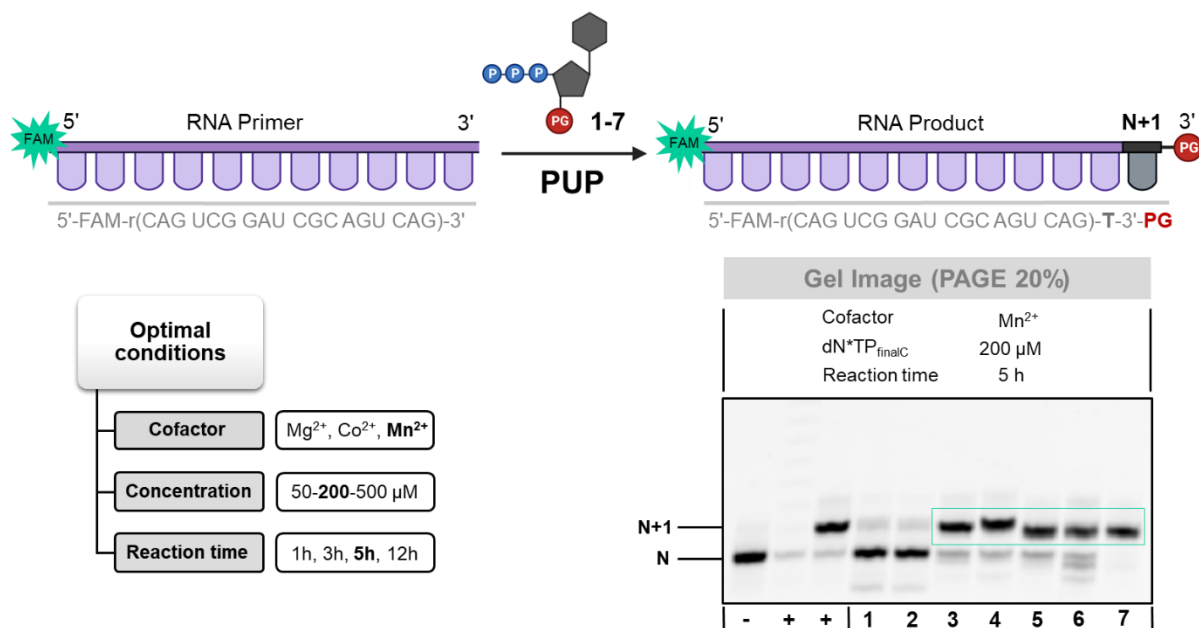


157

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

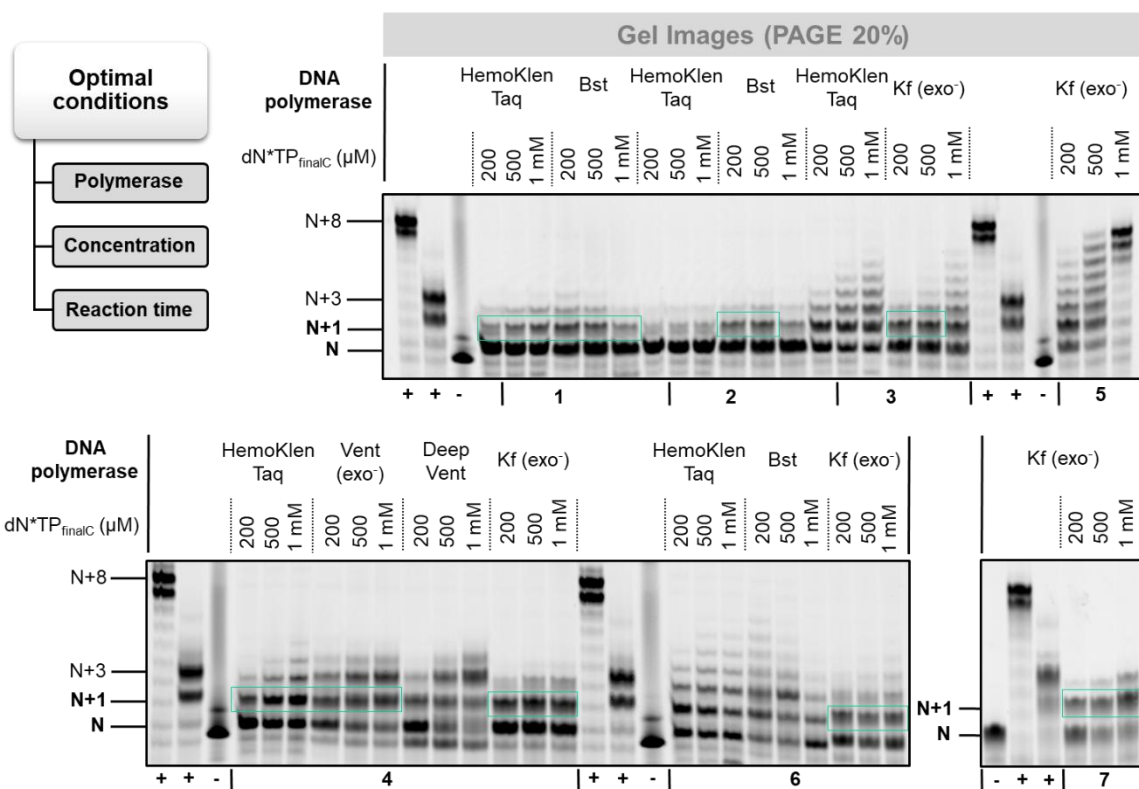
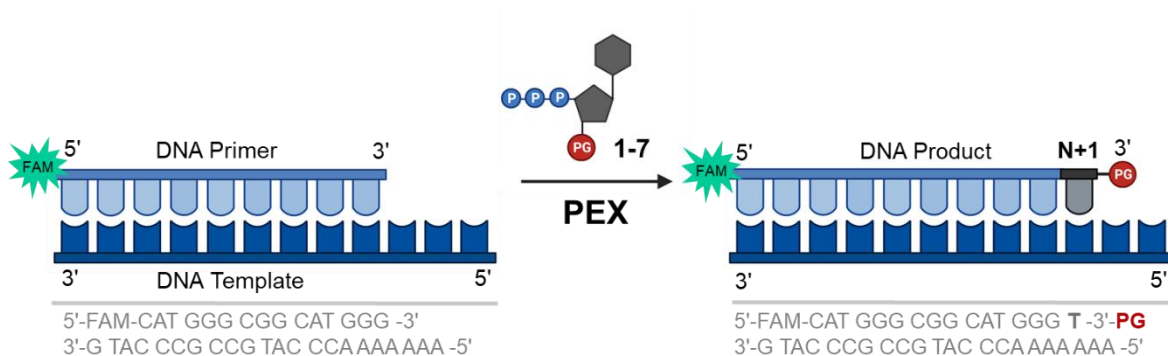
198 While most efforts to improve the efficiency of *de novo* DNA synthesis are centered around TdT-
 199 mediated, template independent oligonucleotide production, template-dependent approaches are also
 200 emerging.(Hoff et al., 2020;Van Giesen et al., 2022;Hoose et al., 2023) A main advantage of
 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*⁻), *Sulfolobus* DNA polymerase IV, (Dpo4), Deep Vent,
211 and Kf (*exo*⁻)) 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*⁻) 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*⁻)
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*⁻) 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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353 **References**

- 354 Aggarwal, T., Hansen, W.A., Hong, J.a.T., Ganguly, A., York, D.M., Khare, S.D., and Izgu, E.C.
355 (2022). Introducing a New Bond-Forming Activity in an Archaeal DNA Polymerase by
356 Structure-Guided Enzyme Redesign. *ACS Chem. Biol.* 17, 1924-1936.
- 357 Andrews, B.I., Antia, F.D., Brueggemeier, S.B., Diorazio, L.J., Koenig, S.G., Kopach, M.E., Lee, H.,
358 Olbrich, M., and Watson, A.L. (2021). Sustainability Challenges and Opportunities in
359 Oligonucleotide Manufacturing. *J. Org. Chem.* 86, 49-61.
- 360 Ashley, J., Potts, I.G., and Olorunniji, F.J. (2023). Applications of Terminal Deoxynucleotidyl
361 Transferase Enzyme in Biotechnology. *ChemBioChem*, 10.1002/cbic.202200510.
- 362 Beaucage, S.L., and Caruthers, M.H. (1981). Deoxynucleoside phosphoramidites—A new class of
363 key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* 22, 1859-1862.
- 364 Bergen, K., Betz, K., Welte, W., Diederichs, K., and Marx, A. (2013). Structures of KOD and 9°N
365 DNA Polymerases Complexed with Primer Template Duplex. *ChemBioChem* 14, 1058-1062.
- 366 Bollum, F.J. (1962). Oligodeoxyribonucleotide-primed Reactions Catalyzed by Calf Thymus
367 Polymerase. *J. Biol. Chem.* 237, 1945-1949.
- 368 Bowers, J., Mitchell, J., Beer, E., Buzby, P.R., Causey, M., Efcavitch, J.W., Jarosz, M., Krzymanska-
369 Olejnik, E., Kung, L., Lipson, D., Lowman, G.M., Marappan, S., Mcinerney, P., Platt, A.,
370 Roy, A., Siddiqi, S.M., Steinmann, K., and Thompson, J.F. (2009). Virtual terminator
371 nucleotides for next-generation DNA sequencing. *Nat. Methods* 6, 593-595.
- 372 Campbell, M.A., and Wengel, J. (2011a). Locked vs. unlocked nucleic acids (LNA vs. UNA):
373 contrasting structures work towards common therapeutic goals. *Chem. Soc. Rev.* 40, 5680-
374 5689.

375 Campbell, M.A., and Wengel, J. (2011b). Locked vs. unlocked nucleic acids (LNAs. UNA):
376 contrasting structures work towards common therapeutic goals. *Chem. Soc. Rev.* 40, 5680-
377 5689.

378 Canard, B., Cardona, B., and Sarfati, R.S. (1995). Catalytic editing properties of DNA polymerases.
379 *Proc. Natl. Acad. Sci. U.S.A.* 92, 10859-10863.

380 Caruthers, M.H. (1985). Gene Synthesis Machines: DNA Chemistry and Its Uses. *Science* 230, 281-
381 285.

382 Chaput, J.C., and Herdewijn, P. (2019). What Is XNA? *Angew. Chem. Int. Ed.* 58, 11570-11572.

383 Chaput, J.C., Herdewijn, P., and Hollenstein, M. (2020). Orthogonal Genetic Systems.
384 *ChemBioChem* 21, 1408-1411.

385 Chaudhary, N., Weissman, D., and Whitehead, K.A. (2021). mRNA vaccines for infectious diseases:
386 principles, delivery and clinical translation. *Nat. Rev. Drug Discov.* 20, 817-838.

387 Chen, F., Dong, M., Ge, M., Zhu, L., Ren, L., Liu, G., and Mu, R. (2013). The History and Advances
388 of Reversible Terminators Used in New Generations of Sequencing Technology. *Genom.*
389 *Proteom. Bioinform.* 11, 34-40.

390 Chen, F., Gaucher, E.A., Leal, N.A., Hutter, D., Havemann, S.A., Govindarajan, S., Ortlund, E.A.,
391 and Benner, S.A. (2010). Reconstructed evolutionary adaptive paths give polymerases
392 accepting reversible terminators for sequencing and SNP detection. *Proc. Natl. Acad. Sci.*
393 *U.S.A.* 107, 1948-1953.

394 Chmiel, C.T., and Long, F.A. (1956). The Hydrolysis of Esters of Some Substituted Benzoic Acids in
395 Strongly Acid Aqueous Solutions. *J. Am. Chem. Soc.* 78, 3326-3330.

396 Choi, H., Choi, Y., Choi, J., Lee, A.C., Yeom, H., Hyun, J., Ryu, T., and Kwon, S. (2022).
397 Purification of multiplex oligonucleotide libraries by synthesis and selection. *Nat. Biotechnol.*
398 40, 47-53.

399 Christensen, N.K., Dahl, B.M., and Nielsen, P. (2001). Incorporation of α - and β -LNA (Locked
400 nucleic acid) monomers in oligodeoxynucleotides with polarity reversals. *Bioorg. Med.*
401 *Chem. Lett.* 11, 1765-1768.

402 Doricchi, A., Platnich, C.M., Gimpel, A., Horn, F., Earle, M., Lanzavecchia, G., Cortajarena, A.L.,
403 Liz-Marzán, L.M., Liu, N., Heckel, R., Grass, R.N., Krahne, R., Keyser, U.F., and Garoli, D.
404 (2022a). Emerging Approaches to DNA Data Storage: Challenges and Prospects. *ACS Nano*
405 16, 17552-17571.

406 Doricchi, A., Platnich, C.M., Gimpel, A., Horn, F., Earle, M., Lanzavecchia, G., Cortajarena, A.L.,
407 Liz-Marzán, L.M., Liu, N., Heckel, R., Grass, R.N., Krahne, R., Keyser, U.F., and Garoli, D.
408 (2022b). Emerging Approaches to DNA Data Storage: Challenges and Prospects. *ACS Nano*,
409 10.1021/acsnano.1022c06748.

410 Dousis, A., Ravichandran, K., Hobert, E.M., Moore, M.J., and Rabideau, A.E. (2022). An engineered
411 T7 RNA polymerase that produces mRNA free of immunostimulatory byproducts. *Nat.*
412 *Biotechnol.*, doi.org/10.1038/s41587-41022-01525-41586.

413 Eichert, A., Behling, K., Betzel, C., Erdmann, V.A., Fürste, J.P., and Förster, C. (2010). The crystal
414 structure of an 'All Locked' nucleic acid duplex. *Nucleic Acids Res.* 38, 6729-6736.

415 Flamme, M., Hanlon, S., Iding, H., Puentener, K., Sladojevich, F., and Hollenstein, M. (2021).
416 Towards the enzymatic synthesis of phosphorothioate containing LNA oligonucleotides.
417 *Bioorg. Med. Chem. Lett.* 48, 128242.

418 Flamme, M., Hanlon, S., Marzuoli, I., Puntener, K., Sladojevich, F., and Hollenstein, M. (2022a).
419 Evaluation of 3'-phosphate as a transient protecting group for controlled enzymatic synthesis
420 of DNA and XNA oligonucleotides. *Commun. Chem.* 5, 68.

421 Flamme, M., Katkevica, D., Pajuste, K., Katkevics, M., Sabat, N., Hanlon, S., Marzuoli, I., Püntener,
422 K., Sladojevich, F., and Hollenstein, M. (2022b). Benzoyl and Pivaloyl as Efficient Protecting

423 Groups for Controlled Enzymatic Synthesis of DNA and XNA Oligonucleotides. *Asian J.*
424 *Org. Chem.* 11, e202200384.

425 Gardner, A.F., Wang, J., Wu, W., Karouby, J., Li, H., Stupi, B.P., Jack, W.E., Hersh, M.N., and
426 Metzker, M.L. (2012). Rapid incorporation kinetics and improved fidelity of a novel class of
427 3'-OH unblocked reversible terminators. *Nucleic Acids Res.* 40, 7404-7415.

428 George, J.T., Azhar, M., Aich, M., Sinha, D., Ambi, U.B., Maiti, S., Chakraborty, D., and Srivatsan,
429 S.G. (2020). Terminal Uridyl Transferase Mediated Site-Directed Access to Clickable
430 Chromatin Employing CRISPR-dCas9. *J. Am. Chem. Soc.* 142, 13954-13965.

431 Guo, J., Xu, N., Li, Z.M., Zhang, S.L., Wu, J., Kim, D.H., Marma, M.S., Meng, Q.L., Cao, H.Y., Li,
432 X.X., Shi, S.D., Yu, L., Kalachikov, S., Russo, J.J., Turro, N.J., and Ju, J.Y. (2008). Four-
433 color DNA sequencing with 3'-O-modified nucleotide reversible terminators and chemically
434 cleavable fluorescent dideoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9145-9150.

435 Gupta, M., Levine, S.R., and Spitale, R.C. (2022). Probing Nascent RNA with Metabolic
436 Incorporation of Modified Nucleosides. *Acc. Chem. Res.* 55, 2647-2659.

437 Hagedorn, P.H., Persson, R., Funder, E.D., Albæk, N., Diemer, S.L., Hansen, D.J., Møller, M.R.,
438 Papargyri, N., Christiansen, H., Hansen, B.R., Hansen, H.F., Jensen, M.A., and Koch, T.
439 (2018). Locked nucleic acid: modality, diversity, and drug discovery. *Drug Discov. Today* 23,
440 101-114.

441 Hajjar, M., Chim, N., Liu, C., Herdewijn, P., and Chaput, John c. (2022). Crystallographic analysis of
442 engineered polymerases synthesizing phosphonomethylthreosyl nucleic acid. *Nucleic Acids*
443 *Res.* 50, 9663-9674.

444 Hoff, K., Halpain, M., Garbagnati, G., Edwards, J.S., and Zhou, W. (2020). Enzymatic Synthesis of
445 Designer DNA Using Cyclic Reversible Termination and a Universal Template. *ACS Synth.*
446 *Biol.* 9, 283-293.

447 Hoose, A., Vellacott, R., Storch, M., Freemont, P.S., and Ryadnov, M.G. (2023). DNA synthesis
448 technologies to close the gene writing gap. *Nat. Rev. Chem.*, 10.1038/s41570-41022-00456-
449 41579.

450 Hoshino, H., Kasahara, Y., Kuwahara, M., and Obika, S. (2020). DNA Polymerase Variants with
451 High Processivity and Accuracy for Encoding and Decoding Locked Nucleic Acid
452 Sequences. *J. Am. Chem. Soc.* 142, 21530-21537.

453 Hutter, D., Kim, M.-J., Karalkar, N., Leal, N.A., Chen, F., Guggenheim, E., Visalakshi, V., Olejnik,
454 J., Gordon, S., and Benner, S.A. (2010). Labeled Nucleoside Triphosphates with Reversibly
455 Terminating Aminoalkoxyl Groups. *Nucleosides, Nucleotides & Nucleic Acids* 29, 879-895.

456 Jackson, N.a.C., Kester, K.E., Casimiro, D., Gurunathan, S., and Derosa, F. (2020). The promise of
457 mRNA vaccines: a biotech and industrial perspective. *npj Vaccines* 5, 11.

458 Jaeger, T., Arsic, M., and Mayer, C. (2005). Scission of the Lactyl Ether Bond of N-Acetylmuramic
459 Acid by *Escherichia coli* "Etherase"*. *J. Biol. Chem.* 280, 30100-30106.

460 Jang, E.K., Son, R.G., and Pack, S.P. (2019). Novel enzymatic single-nucleotide modification of
461 DNA oligomer: prevention of incessant incorporation of nucleotidyl transferase by
462 ribonucleotide-borate complex. *Nucleic Acids Res.* 47.

463 Jensen, M.A., and Davis, R.W. (2018). Template-Independent Enzymatic Oligonucleotide Synthesis
464 (TiEOS): Its History, Prospects, and Challenges. *Biochemistry* 57, 1821-1832.

465 Ju, J.Y., Kim, D.H., Bi, L.R., Meng, Q.L., Bai, X.P., Li, Z.M., Li, X.X., Marma, M.S., Shi, S., Wu,
466 J., Edwards, J.R., Romu, A., and Turro, N.J. (2006). Four-color DNA sequencing by synthesis
467 using cleavable fluorescent nucleotide reversible terminators. *Proc. Natl. Acad. Sci. U.S.A.*
468 103, 19635-19640.

469 Jung, H.S., Jung, W.B., Wang, J., Abbott, J., Horgan, A., Fournier, M., Hinton, H., Hwang, Y.H.,
470 Godron, X., Nicol, R., Park, H., and Ham, D. (2022). CMOS electrochemical pH localizer-
471 imager. *Sci. Adv.* 8.

472 K. Singh, S., A. Koshkin, A., Wengel, J., and Nielsen, P. (1998). LNA (locked nucleic acids):
473 synthesis and high-affinity nucleic acid recognition. *Chem. Commun.*, 455-456.

474 Kasahara, Y., Kitadume, S., Morihira, K., Kuwahara, M., Ozaki, H., Sawai, H., Imanishi, T., and
475 Obika, S. (2010). Effect of 3'-end capping of aptamer with various 2',4'-bridged nucleotides:
476 Enzymatic post-modification toward a practical use of polyclonal aptamers. *Bioorg. Med.*
477 *Chem. Lett.* 20, 1626-1629.

478 Keller, A.C., Serva, S., Knapp, D.C., Kwiatkowski, M., and Engels, J.W. (2009). Synthesis of 3'-O-
479 (2-cyanoethyl)-2'-deoxythymidine-5'-phosphate as a model compound for evaluation of
480 cyanoethyl cleavage. *Collect. Czechoslov. Chem. Commun.* 74, 515-534.

481 Knapp, D.C., Serva, S., D'onofrio, J., Keller, A., Lubys, A., Kurg, A., Remm, M., and Engels, J.W.
482 (2011). Fluoride-Cleavable, Fluorescently Labelled Reversible Terminators: Synthesis and
483 Use in Primer Extension. *Chem. Eur. J.* 17, 2903-2915.

484 Krayevsky, A.A., Victorova, L.S., Arzumanov, A.A., and Jasko, M.V. (2000). Terminal
485 deoxynucleotidyl transferase: catalysis of DNA (oligodeoxynucleotide) phosphorylation.
486 *Pharmacol. Ther.* 85, 165-173.

487 Kuwahara, M., Obika, S., Takeshima, H., Hagiwara, Y., Nagashima, J.-I., Ozaki, H., Sawai, H., and
488 Imanishi, T. (2009). Smart conferring of nuclease resistance to DNA by 3'-end protection
489 using 2',4'-bridged nucleoside-5'-triphosphates. *Bioorg. Med. Chem. Lett.* 19, 2941-2943.

490 Kwak, J.E., and Wickens, M. (2007). A family of poly(U) polymerases. *RNA* 13, 860-867.

491 Larsen, A.C., Dunn, M.R., Hatch, A., Sau, S.P., Youngbull, C., and Chaput, J.C. (2016). A general
492 strategy for expanding polymerase function by droplet microfluidics. *Nat. Commun.* 7, 11235.

493 Lee, H.H., Kalhor, R., Goela, N., Bolot, J., and Church, G.M. (2019a). Terminator-free template-
494 independent enzymatic DNA synthesis for digital information storage. *Nat. Commun.* 10.

495 Lee, H.H., Kalhor, R., Goela, N., Bolot, J., and Church, G.M. (2019b). Terminator-free template-
496 independent enzymatic DNA synthesis for digital information storage. *Nat. Commun.* 10,
497 2383.

498 Lindenburg, L., Huovinen, T., Van de wiel, K., Herger, M., Snaith, M.R., and Hollfelder, F. (2020).
499 Split & mix assembly of DNA libraries for ultrahigh throughput on-bead screening of
500 functional proteins. *Nucleic Acids Res.* 48, e63-e63.

501 Linwu, S.-W., Tsai, T.-Y., Tu, Y.-H., Chi, H.-W., Tsao, Y.-P., Chen, Y.-C., Wang, H.-M., Chang,
502 W.-H., Chiou, C.-F., Lee, J., and Chen, C.-Y. (2020). Enzymatic Cleavage of 3'-Esterified
503 Nucleotides Enables a Long, Continuous DNA Synthesis. *Sci. Rep.* 10, 7515.

504 Linwu, S.-W., Tu, Y.-H., Tsai, T.-Y., Maestre-Reyna, M., Liu, M.-S., Wu, W.-J., Huang, J.-Y., Chi,
505 H.-W., Chang, W.-H., Chiou, C.-F., Wang, A.H.J., Lee, J., and Tsai, M.-D. (2019).
506 *Thermococcus* sp. 9^oN DNA polymerase exhibits 3'-esterase activity that can be harnessed
507 for DNA sequencing. *Commun. Biol.* 2, 224.

508 Liu, A., and Wang, X. (2022). The Pivotal Role of Chemical Modifications in mRNA Therapeutics.
509 *Front. Cell Dev. Biol.* 10.

510 Lu, X., Li, J., Li, C., Lou, Q., Peng, K., Cai, B., Liu, Y., Yao, Y., Lu, L., Tian, Z., Ma, H., Wang, W.,
511 Cheng, J., Guo, X., Jiang, H., and Ma, Y. (2022). Enzymatic DNA Synthesis by Engineering
512 Terminal Deoxynucleotidyl Transferase. *ACS Catal.* 12, 2988-2997.

513 Ludwig, J., and Eckstein, F. (1989). Rapid and efficient synthesis of nucleoside 5'-O-(1-
514 thiotriphosphates), 5'-triphosphates and 2',3'-cyclophosphorothioates using 2-chloro-4H-1,3,2-
515 benzodioxaphosphorin-4-one. *J. Org. Chem.* 54, 631-635.

516 Mackey, J.K., and Gilham, P.T. (1971). New Approach to the Synthesis of Polyribonucleotides of
517 Defined Sequence. *Nature* 233, 551-553.

518 Masaki, Y., Onishi, Y., and Seio, K. (2022). Quantification of synthetic errors during chemical
519 synthesis of DNA and its suppression by non-canonical nucleosides. *Sci. Rep.* 12, 12095.

520 Mathews, A.S., Yang, H., and Montemagno, C. (2016). Photo-cleavable nucleotides for primer free
521 enzyme mediated DNA synthesis. *Org. Biomol. Chem.* 14, 8278-8288.

522 Matthey-Doret, C., Colp, M.J., Escoll, P., Thierry, A., Moreau, P., Curtis, B., Sahr, T., Sarrasin, M.,
523 Gray, M.W., Lang, B.F., Archibald, J.M., Buchrieser, C., and Koszul, R. (2022).
524 Chromosome-scale assemblies of *Acanthamoeba castellanii* genomes provide insights into
525 *Legionella pneumophila* infection-related chromatin reorganization. *Genome Res.* 32, 1698-
526 1710.

527 Mckenzie, L.K., El-Khoury, R., Thorpe, J.D., Damha, M.J., and Hollenstein, M. (2021). Recent
528 progress in non-native nucleic acid modifications. *Chem. Soc. Rev.* 50, 5126-5164.

529 Molina, A.G., and Sanghvi, Y.S. (2019). Liquid-Phase Oligonucleotide Synthesis: Past, Present, and
530 Future Predictions. *Curr. Protoc. Nucleic Acid Chem.* 77, e82.

531 Munoz-Tello, P., Gabus, C., and Thore, S. (2012). Functional Implications from the Cid1 Poly(U)
532 Polymerase Crystal Structure. *Structure* 20, 977-986.

533 Nance, K.D., and Meier, J.L. (2021). Modifications in an Emergency: The Role of N1-
534 Methylpseudouridine in COVID-19 Vaccines. *ACS Cent. Sci.* 7, 748-756.

535 Obika, S., Nanbu, D., Hari, Y., Andoh, J.-I., Morio, K.-I., Doi, T., and Imanishi, T. (1998). Stability
536 and structural features of the duplexes containing nucleoside analogues with a fixed N-type
537 conformation, 2'-O,4'-C-methyleneribonucleosides. *Tetrahedron Lett.* 39, 5401-5404.

538 Palla, M., Guo, W.J., Shi, S.D., Li, Z.M., Wu, J., Jockusch, S., Guo, C., Russo, J.J., Turro, N.J., and
539 Ju, J.Y. (2014). DNA sequencing by synthesis using 3'-O-azidomethyl nucleotide reversible
540 terminators and surface-enhanced Raman spectroscopic detection. *RSC Adv.* 4, 49342-49346.

541 Palluk, S., Arlow, D.H., De Rond, T., Barthel, S., Kang, J.S., Bector, R., Baghdassarian, H.M.,
542 Truong, A.N., Kim, P.W., Singh, A.K., Hillson, N.J., and Keasling, J.D. (2018). De novo
543 DNA synthesis using polymerase-nucleotide conjugates. *Nat. Biotechnol.* 36, 645-650.

544 Parsons, J.F., Calabrese, K., Eisenstein, E., and Ladner, J.E. (2003). Structure and Mechanism of
545 *Pseudomonas aeruginosa* PhzD, an Isochorismatase from the Phenazine Biosynthetic
546 Pathway. *Biochemistry* 42, 5684-5693.

547 Pengthong, P., Bopp, P.A., Jungsuttiwong, S., and Nanok, T. (2023). Mechanistic insights into the
548 self-esterification of lactic acid under neutral and acidic conditions. *J. Mol. Struct.* 1273,
549 134336.

550 Picart, P., Domínguez De María, P., and Schallmeyer, A. (2015). From gene to biorefinery: microbial
551 β -etherases as promising biocatalysts for lignin valorization. *Front. Microbiol.* 6.

552 Pinheiro, V.B., Taylor, A.I., Cozens, C., Abramov, M., Renders, M., Zhang, S., Chaput, J.C.,
553 Wengel, J., Peak-Chew, S.-Y., Mclaughlin, S.H., Herdewijn, P., and Holliger, P. (2012).
554 Synthetic Genetic Polymers Capable of Heredity and Evolution. *Science* 336, 341-344.

555 Ruparel, H., Bi, L.R., Li, Z.M., Bai, X.P., Kim, D.H., Turro, N.J., and Ju, J.Y. (2005). Design and
556 synthesis of a 3'-O-allyl photocleavable fluorescent nucleotide as a reversible terminator for
557 DNA sequencing by synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5932-5937.

558 Sarac, I., and Hollenstein, M. (2019). Terminal Deoxynucleotidyl Transferase in the Synthesis and
559 Modification of Nucleic Acids. *ChemBioChem* 20, 860-871.

560 Shi, H., Wang, Y., and Hua, R. (2015). Acid-catalyzed carboxylic acid esterification and ester
561 hydrolysis mechanism: acylium ion as a sharing active intermediate via a spontaneous
562 trimolecular reaction based on density functional theory calculation and supported by
563 electrospray ionization-mass spectrometry. *Phys. Chem. Chem. Phys.* 17, 30279-30291.

564 Statello, L., Guo, C.-J., Chen, L.-L., and Huarte, M. (2021). Gene regulation by long non-coding
565 RNAs and its biological functions. *Nat. Rev. Mol. Cell Biol.* 22, 96-118.

566 Taylor, A.I., Beuron, F., Peak-Chew, S.-Y., Morris, E.P., Herdewijn, P., and Holliger, P. (2016).
567 Nanostructures from Synthetic Genetic Polymers. *ChemBioChem* 17, 1107-1110.

- 568 Van Giesen, K.J.D., Thompson, M.J., Meng, Q., and Lovelock, S.L. (2022). Biocatalytic Synthesis of
569 Antiviral Nucleosides, Cyclic Dinucleotides, and Oligonucleotide Therapies. *JACS Au*,
570 10.1021/jacsau.1022c00481.
- 571 Veedu, R.N., Vester, B., and Wengel, J. (2010). Polymerase directed incorporation studies of LNA-G
572 nucleoside 5'-triphosphate and primer extension involving all four LNA nucleotides. *New J.*
573 *Chem.* 34, 877-879.
- 574 Venter, J.C., Glass, J.I., Hutchison, C.A., and Vashee, S. (2022). Synthetic chromosomes, genomes,
575 viruses, and cells. *Cell* 185, 2708-2724.
- 576 Vo, J.M., Mulrone, L., Quick-Cleveland, J., Jain, M., Akeson, M., and Ares, M. (2021). Synthesis
577 of modified nucleotide polymers by the poly(U) polymerase Cid1: application to direct RNA
578 sequencing on nanopores. *RNA* 27, 1497-1511.
- 579 Vummidi, B.R., Farrera-Soler, L., Dagher, J.-P., Dockerill, M., Barluenga, S., and Winssinger, N.
580 (2022). A mating mechanism to generate diversity for the Darwinian selection of DNA-
581 encoded synthetic molecules. *Nat. Chem.* 14, 141-152.
- 582 Wang, G., He, C., Zou, J., Liu, J., Du, Y., and Chen, T. (2022). Enzymatic Synthesis of DNA with an
583 Expanded Genetic Alphabet Using Terminal Deoxynucleotidyl Transferase. *ACS Syn. Biol.*
584 11, 4142-4155.
- 585 Winz, M.L., Samanta, A., Benzinger, D., and Jaschke, A. (2012). Site-specific terminal and internal
586 labeling of RNA by poly(A) polymerase tailing and copper-catalyzed or copper-free strain-
587 promoted click chemistry. *Nucleic Acids Res.* 40.
- 588 Wu, J., Zhang, S.L., Meng, Q.L., Cao, H.Y., Li, Z.M., Li, X.X., Shi, S.D., Kim, D.H., Bi, L.R.,
589 Turro, N.J., and Ju, J.Y. (2007). 3'-O-modified nucleotides as reversible terminators for
590 pyrosequencing. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16462-16467.
- 591 Zuckerman, B., Ron, M., Mikl, M., Segal, E., and Ulitsky, I. (2020). Gene Architecture and Sequence
592 Composition Underpin Selective Dependency of Nuclear Export of Long RNAs on NXF1 and
593 the TREX Complex. *Mol. Cell* 79, 251-267.e256.

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