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COMMUNICATION

Compatibility of 5-ethynyl-2'F-ANA UTP with *in vitro* selection for the generation of base-modified, nuclease resistant aptamers

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A modified nucleoside triphosphate bearing two modifications based on a 2'-deoxy-2'-fluoro-arabinofuranose sugar and a uracil nucleobase equipped with a C5-ethynyl moiety (5-ethynyl-2'F-ANA UTP) was synthesized. This nucleotide analog could enzymatically be incorporated into DNA oligonucleotides by primer extension and reverse transcribed to unmodified DNA. . This nucleotide could be used in SELEX for the identification of high binding affinity and nuclease resistant aptamers.

Xeno nucleic acids (XNAs) are analogs of the two natural genetic polymers (DNA and RNA) bearing modifications at the level of the sugar.[1-4] The presence of these chemical modifications provides XNAs with properties that are absent or muted in corresponding natural systems. For instance, the presence of sugar modifications markedly improves the nuclease resistance of oligonucleotides which is of highly relevant for therapeutic[5-9] and *in vivo* applications.[10, 11] Similarly, base-modifications convey orthogonality to modified nucleic acids[12-19] and provide functionalities to synthetic genetic polymers, which improve their catalytic[20-22] and/or binding capacities.[23-25] The synthesis of XNAs proceeds either via a chemical route (using phosphoramidites during automated solid-phase synthesis) or a chemoenzymatic method, whereby a polymerase incorporates modified nucleotides.[26, 27] The latter method endows some advantages compared to solid phase synthesis, including: 1) high functional group tolerance; 2) unrestricted oligonucleotide length; and 3) compatibility with SELEX and related combinatorial *in vitro* selection methods. Combined with recent progress in polymerase engineering[28] these intrinsic properties have instigated the selection of numerous XNA aptamers[29-37] and catalysts.[38-40] However, the modification pattern of most XNA nucleotide analogues is restricted to a single location of the nucleosidic scaffold. To date, only a few reports have shown the possibility of introducing two simultaneous modifications in XNAs, all concerning threose nucleic acid (TNA) nucleotides[32, 41, 42]. Here, we report the synthesis and biochemical characterization of a 2'-deoxy-2'F-arabinonucleic acid (2'F-ANA) uridine analog decorated with an ethynyl group on the nucleobase. We clearly demonstrate the compatibility of this XZA nucleotide (modification of the sugar (X instead of D) and the nucleobase (Z instead of N))[4] with a full replication cycle (i.e. DNA-dependent synthesis of modified DNA followed by reverse-transcription to wild-type DNA) and thus with SELEX methods. Finally, we also demonstrate the usefulness of the ethynyl moiety as a synthetic handle for the further modification of XNA polymers.

The 2'F-ANA modification is known to increase the affinity of DNA for complementary RNA, is a DNA mimic (South/East sugar pucker) and increases the nuclease resistance of oligonucleotides,[43-46] thus it is worthwhile to pursue. Moreover, the 2'F-ANA scaffold is clearly compatible with SELEX since 2'F-ANA 5'-triphosphates are substrates for DNA polymerases,[47, 48] and recently, potent catalysts[38, 39] and aptamers[30, 31] were isolated. The 5-ethynyl-dUTP (EdUTP) base modification is a widely used nucleotide analog.[24, 49] It is well tolerated by polymerases *in vitro* and *in vivo,*[50-52] and the alkyne moiety represents a convenient synthetic handle to incorporate additional modifications by applying click chemistry (copper(I)-catalyzed alkyne–azide cycloaddition or CuAAC).[53]

Synthesis of the modified nucleotide started with the bromination of the anomeric center of the commercially available nucleoside **1** (Scheme 1). The presence of the 2'-fluorine ensures that the bromine atom is installed in the α configuration in compound **2**. The expected South pucker of arabinose places the halogens at C1' and C2' in pseudoaxial positions, which are stabilized by gauche (O4'-C1'-C2'-F2') and anomeric effects (O4'-C1'-Br), respectively.[54] The modified

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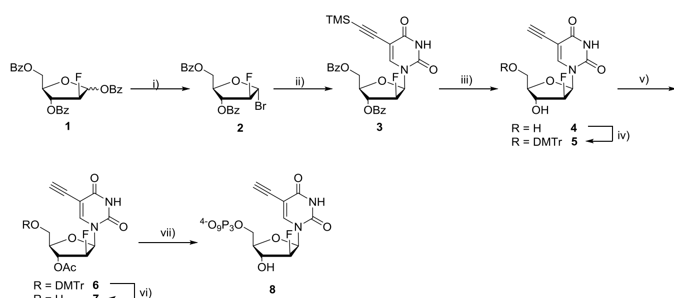
nucleobase was then installed on the sugar moiety by application of modified Vorbrüggen conditions.[55, 56] This reaction is always in competition with the analogous S_N1 reaction, which forms a mixture of both anomers. The contribution of S_N1 and S_N2 depends mostly on the solvent and the temperature, and generally the combination of low solvent polarity and elevated temperature renders acceptable results. We therefore chose toluene and 80°C overnight reflux as reaction conditions (see Supporting Information) and both anomers were formed (1:10 α/β), which could be separated by chromatography yielding compound **3** in moderate yields. Removal of the blocking groups of **3** was followed by 5'-DMTr protection yielding **5** in good yields (57% over two steps). Acetylation and detritylation of nucleoside **5** led to the suitably 3'-*O*-protected nucleoside **7**, which was subjected to Ludwig-Eckstein conditions[57] to generate the desired 5'-triphosphate **8**. The low yields obtained during this last step stem from a rather lengthy purification protocol and highlight the need for more robust and generally applicable protocols for the synthesis of nucleoside triphosphate analogs.[58]



Scheme 1. Synthesis of 5-ethynyl-2'F-ANA UTP **8**. Reagents and conditions: i) HBr/AcOH, 0°C to rt, 12 h; ii) 5-(2-trimethylsilylethynyl)-uracil, BSA, toluene, 80°C, 12h, 42% (over 2 steps); iii) a) NaOH in THF/MeOH/H_2O (5:4:1), 0°C, 1h; b) NH_4Cl, rt, 20 min, 69%; iv) DMTrCl, pyridine, rt, 12 h, 83%; v) Ac_2O, pyridine, 0°C to rt, 1h, 80%; vi) TFA, CH_2Cl_2, 0°C to rt, 30 min, 45%; vii) a) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; b) $(nBu_3NH)_2H_2P_2O_7$, DMF, nBu_3N, rt, 45 min; c) I_2, pyridine, H_2O, rt, 30 min; d) NH_3(aq.), rt, 1.5 h, 2% (4 steps).

With 5-ethynyl-2'F-ANA UTP **8** at hand, we proceeded to evaluate the capacity of polymerases to incorporate this modified nucleotide into DNA. To do so, primer extension (PEX) reactions were carried out with various polymerases (Therminator, Vent (*exo*$^-$), *Bst*, Deep Vent, the Klenow fragment of DNA polymerase I *exo*$^-$ (Kf *exo*$^-$), and Taq) and by using triphosphate **8** instead of its natural counterpart dTTP. The possibility of incorporating nucleotide **8** was first evaluated using the 37-nucleotide long template **T1** and the 5'-FAM-labelled, 21-mer primer **P1** (see Table S1 for the sequence compositions). With the exception of *Bst*, all polymerases led to the full conversion of primers to the expected full-length products with minimal truncated sequences (Figure S1). Next, we evaluated the possibility of incorporating multiple, consecutive modifications. To this end, we applied template **T2**, which contains seven adenine nucleotides located immediately 5'-downstream to the 3'-end of primer **P2**.[59] In this context, Vent (*exo*$^-$) polymerase managed to incorporate six modified nucleotides while Therminator produced full length products accompanied by some non-templated addition (Figure S2).[60] Moreover, Deep Vent, Taq, and Kf *exo*$^-$ also incorporated the modified nucleotide albeit less efficiently, while *Bst* did not incorporate any modified nucleotide. A similar preference for family B over family A polymerases was observed previously with unmodified 2'F-ANA-nucleotides, suggesting that the ethynyl moiety has a negligible impact on the incorporation efficiency.[47, 48] Surprisingly, nucleotide **8** is a rather poor substrate for polymerases under PCR conditions since only Therminator led to amplified full-length products albeit in lower yields compared to the control reaction performed with natural dNTPs (Figure S4).

Having identified conditions, which enable the polymerization of the modified nucleotide, we wanted to ascertain that the ethynyl moiety, once installed in 2'F-ANA-modified oligonucleotides, the ethynyl moiety was still accessible to further chemical modification by the CuAAC reaction. Thus, PEX reactions were carried out with the **T3**/**P3** system. The resulting products were then incubated with an azide-modified biotin derivative (obtained by amide coupling of biotin with 2-azidoethanamine[61]) under click reaction conditions (see Supporting Information). Next, the biotin-modified, 5-ethynyl-2'F-ANA -containing oligonucleotides were incubated with avidin and analyzed using gel electrophoresis.[42] Figure 1 clearly shows that biotinylated avidin treated 2'F-ANA-oligonucleotides from Therminator PEX reactions are unable to enter the gel due to the presence of the protein (lane 8). A similar trend can be observed when using Vent (*exo*$^-$) as a polymerase, albeit in lower yields (lane 7).

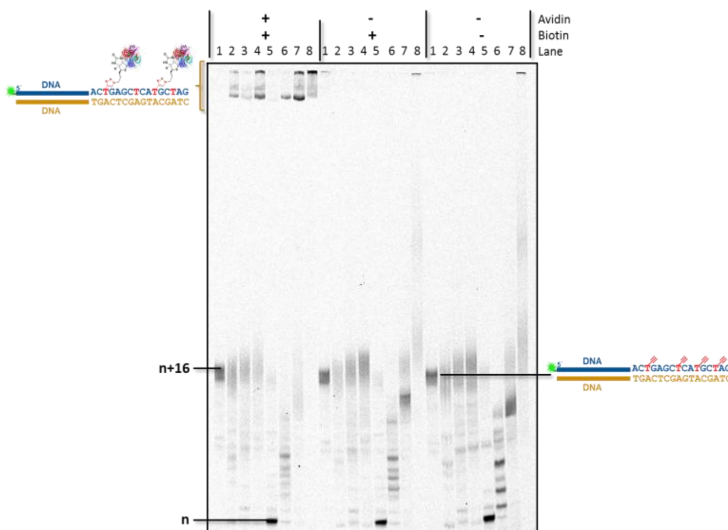


Figure 1. Gel analysis (PAGE 20%) of the functionalization of 2'F-ANA-oligonucleotides by the CuAAC reaction. PEX reactions were carried out with the 31-mer long template **T3** and the 15-mer primer **P3** leading to oligonucleotides containing four 5-ethynyl-2'F-ANA nucleotides. Lanes 1: PEX reactions with natural dNTPs and Taq polymerase; lanes 2: T4 DNA polymerase; lanes 3: Kf *exo*$^-$; lanes 4: *Bst*; lanes 5: Taq polymerase; lanes 6: Deep Vent; lanes 7: Vent (*exo*$^-$); lanes 8: Therminator.

Lastly, to show applicability for SELEX experiments, it was necessary to demonstrate that: 1) modified DNA synthesis did not encounter misincorporation events; and 2) sequences

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containing 5-ethynyl-2'F-ANA-U modifications could be reverse-transcribed back to unmodified DNA. To do so, first, the PEX reactions proceeded with a 98-nucleotide long template **T4** and a 25-mer primer **P4** (Figure S3).[60] Next, λ-exonuclease was applied to digest and remove the unmodified template strand and the modified strand served as a template for DNA synthesis under PCR conditions (Figure 2A). Of the different polymerases tested, Q5 DNA polymerase led to strong amplification of the modified template. The resulting dsDNA product was then introduced into a library, which was subjected to Illumina sequencing (see Supporting Information). Data processing involved phix removal, mapping the data on the sense strand, and analysing the concordance (Figure 2B) as well as the number of errors per read (Figure S4). This analysis showed that 99.2% of the reads have a concordance > 95% and that 95% of all reads have 2 or less errors with no detectable single nucleotide variants (SNVs) in any of the reads.[62] Lastly, the very low rate of base substitution (<0.5%) further confirms the high fidelity of the DNA-modified DNA-DNA cycle.

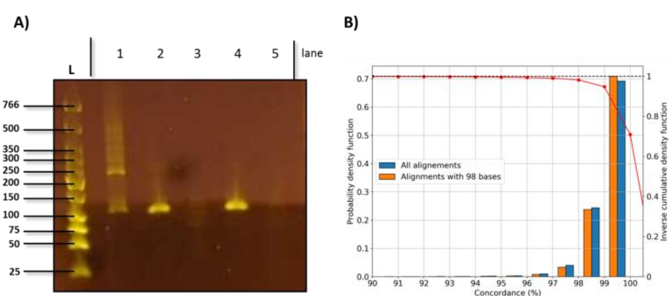

Figure 2. A) Gel analysis (agarose 2%) of reverse-transcription reactions using a 98 nucleotide long FANA-modified template and natural dNTPs. Lane 1: Phusion DNA polymerase and modified template; lane 2: Q5 DNA polymerase and modified template; lane 3: KOD XL DNA polymerase and modified template; lane 4: Taq DNA polymerase and unmodified DNA template; lane 5: Taq DNA polymerase, unmodified DNA template, and only three natural dNTPs (dATP, dCTP, dGTP). L represents a DNA weight ladder. B) Concordance of all the reads mapped to the Q5 DNA polymerase; concordance is defined as 1 - (D+I+M) / (D+M+I+S) where D, I, S and M stands for deletion, insertion, substitution and match, respectively)

Conclusions

In this report, we produced an XZA nucleoside triphosphate bearing two modifications consisting of a 2'F-ANA-sugar moiety and a C5-modified nucleobase. We also highlighted its compatibility with enzymatic synthesis of modified DNA and the possibility of using the ethynyl anchor for the introduction of additional functional groups. Lastly, sequencing and data analysis revealed the fidelity of incorporating the modified nucleotide into DNA and the efficiency of the conversion of 2'F-ANA-containing oligonucleotides back to DNA. The efficiency of 5-ethynyl-2'F-ANA-synthesis might be further improved by using engineered polymerases[31] and the sequencing protocol simplified by application of nanopore sequencing.[63] Work towards these aims as well as application of the click-SELEX protocol[24, 64] to identify aptamers featuring high binding affinity and nuclease resistance is currently ongoing.

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Conflicts of interest

There are no conflicts to declare.

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