Bioconjugate Chemistry

Communication

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Synthesis of 2′-*O*-Propargyl Nucleoside Triphosphates for Enzymatic Oligonucleotide Preparation and "Click" Modification of DNA with Nile Red as Fluorescent Probe

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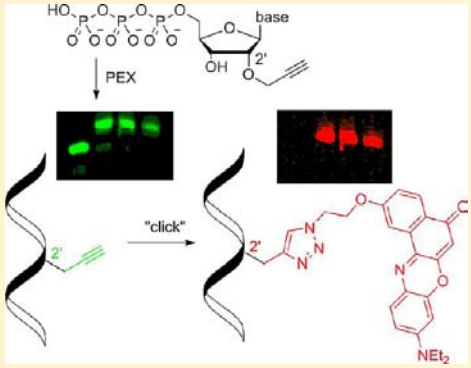
Supporting Information

ABSTRACT: Uridine, adenosine, guanosine, and cytidine that carry a propargyl group attached to the 2′-oxygen were converted efficiently to the corresponding nucleoside triphosphates (pNTPs). Primer extension experiments revealed that pUTP, pATP, and pGTP can be successfully incorporated in oligonucleotides in the so-called 9°N and Therminator DNA polymerases. Most importantly, the ethynyl group as single 2′-modification of the enzymatically prepared oligonucleotides can be applied for postsynthetic labeling. This was representatively shown by PAGE analysis after the "click"-type cycloaddition with the fluorescent nile red azide. These results show that the 2′-position as one of the most important modification sites in oligonucleotides is now accessible not only for synthetic, but also for enzymatic oligonucleotide preparation.



INTRODUCTION

The fast, efficient, and reliable preparation of fluorescently labeled DNA and RNA is a topic of continuously important interest for both chemical bioanalytics and fluorescent cell imaging.[1−3] In particular, the enzymatic synthesis of fluorescent DNA pieces represents a growing research area.[4−8] The application of bioorthogonal and especially "click"-type ligations has been established over the past decade not only to introduce single labels, but also for sequential labeling of oligonucleotides.[9,10] This has been achieved primarily by making synthetically available the corresponding phosphoramidites as building blocks for automated DNA synthesis. Alternatively, providing modified nucleoside triphosphates has the advantage that the subsequent DNA polymerase-assisted preparation of oligonucleotides works under very mild conditions and allows in principle the synthesis of longer and highly modified sequences by PCR.[11,12] Corresponding 2′-deoxynucleoside triphosphates (dNTPs) that are modified at the base moiety have been tested successfully in primer extension (PEX) and PCR experiments.[13−15] Sugar-modified NTPs, especially those carrying the reactive group at the 2′-position, often fail to be accepted as substrates for DNA polymerases since they are recognized as RNA monomers.[16,17] We recently published "click"-type modifications at 2′-*O*-propargylated uridines that were incorporated synthetically into oligonucleotides using the corresponding phosphoramidite as a building block.[18−21] The advantage of 2′-labeling is that the thereby modified uridine in DNA is still able to recognize adenine in the counterstrand.[18] Hence, such modified duplexes exhibit thermal stabilities that are comparable to that of unmodified duplexes. Moreover, attachment to the 2′-position places the fluorophore into the minor groove and thereby promotes significant interactions with the DNA base stack. Herein, we present the preparation of four pNTPs **1**−**4** carrying a propargyl group in the 2′-position, their application in PEX experiments, and the subsequent "click"-type modification with the nile red azide **5**.[22,23]

RESULTS AND DISCUSSION

The unprotected 2′-*O*-propargyl nucleosides can be directly converted to the corresponding triphosphates using the procedure by Ludwig[24] (Scheme 1). Proton sponge prevents hydrochlorination of the ethynyl group.[25] Subsequent treatment with pyrophosphate in dry DMF yields the triphosphates which were purified by ion exchange and reversed phase chromatography. The 2′-*O*-propargylated pNTPs **1**−**4** were obtained in yields between 14% and 35%.

The first primer extension experiments were performed representatively with 2′-*O*-propargylated uridine (**1**, pUTP) using the standing start setup consisting of a primer with 23 nucleotides and template 1 that bears additional 12 nucleotides (Scheme 1). We tested the 9°N_m DNA polymerase from *E. coli* and its so-called Therminator mutants.[26] 9°N polymerase belongs to the B family and has been applied for structurally similar substrates.[17,27] In these DNA polymerases, the sugar "steric gate" of normal DNA polymerases is unlocked.[16] The mutants differ in the accessibility of the active site for 2′-modified NTPs. Accordingly, the experiments with template 1

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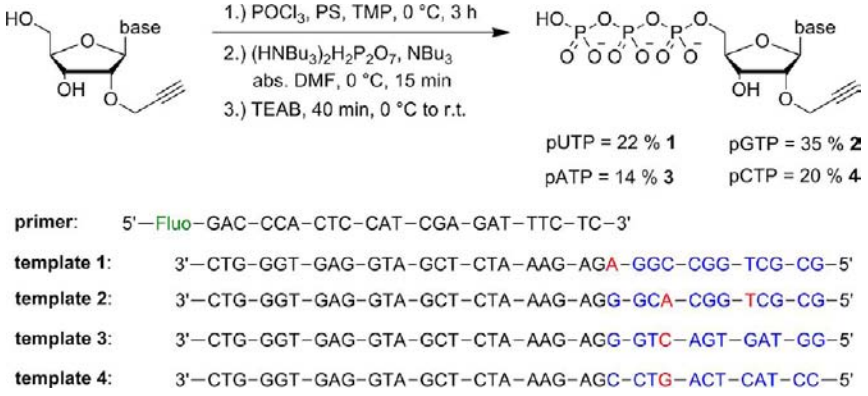
Scheme 1. Synthesis of 2′-*O*-Propargyl Nucleoside Triphosphates 1−4 (pNTPs) and Sequences of Primer, Template 1 for Standing Start Experiment and Templates 2-4 for Running Start Experiments[a]



[a]The primer was labeled with fluorescein (see Figure S2) for gel imaging.

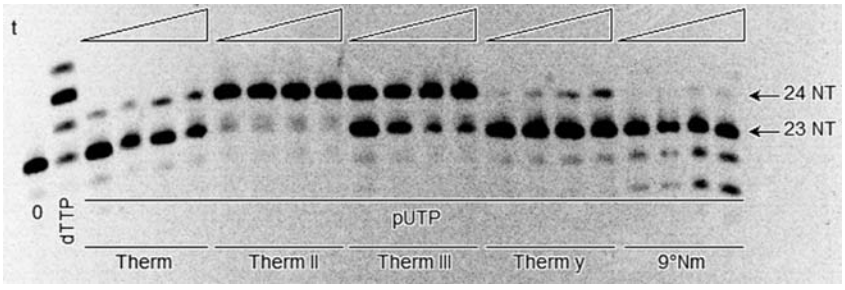


Figure 1. PAGE of standing start primer extension with pUTP and template 1: Primer (750 nM), template (900 nM), polymerase (0.2 U), TTP or pUTP (200 μM), 59 °C, t = 5, 10, 20, 30 min. Lane 0: no pUTP/TTP; lane 0 and lane TTP were stopped after 5 min.

revealed that all four DNA polymerases are able to extend the primer with pUTP but with differing activities (Figure 1). Especially, conversion in the presence of 9°N_m is so slow that even the significantly reduced 3′-5′ exonuclease activity of this mutant cleaves the primer. Therm II and Therm III show highest enzymatic activity and hence most convincing results.

According to the first results, Therm II and Therm III were used in the following running start experiment with template 2 (Scheme 1). The key step is the incorporation of pUTP opposite from A in position 27. PAGE analysis reveals that primer extension with all four natural dNTPs is completed in 45 s (Figure 2). The additional slow-moving bands in the gel images can be assigned to template-free, further elongation, which is a known phenomenon.[28] The control experiment with dATP, dGTP, and dCTP, but without TTP or pUTP, reveals misincorporation opposite from A since the primer is elongated to full-length oligonucleotide within seconds. If TTP is replaced by the 2′-modified pUTP, primer extension takes significantly longer and is—in the case of Therm III—completed after 5 h. Hence, the slow time scale of the primer extension in the presence of pUTP indicates that the modified nucleotide is indeed correctly inserted. Additionally, the small, but observable, gel shifts between the bands of the primer extended with TTP and that with pUTP (as marked in Figure 2) indicates successful modification.

These results provide a promising basis to test the incorporation of the other three 2′-modified pNTPs by the more effective Therm III under slightly modified reaction conditions. Template 2 was used for both pUTP and pATP, since a single A and a single T are present as exclusive incorporation sites at positions 27 and 31, respectively. For

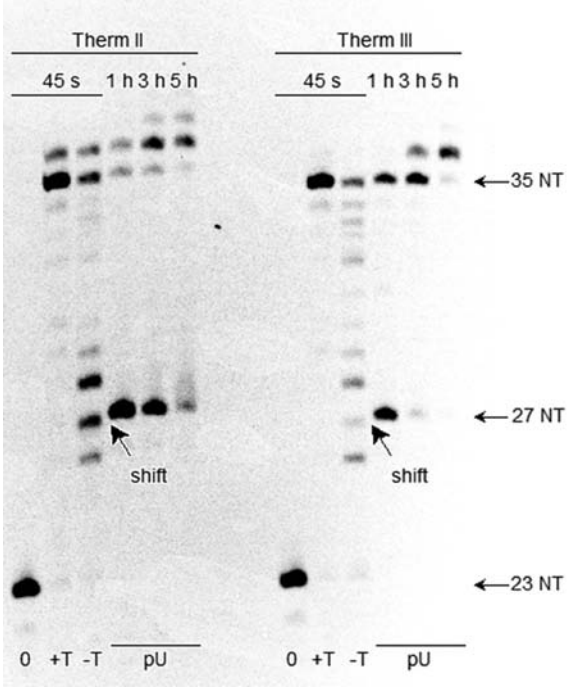


Figure 2. PAGE of running start primer extension with Therm II/Therm III and template 2: Primer (750 nM), template (900 nM), polymerase (2.0 U), dATP, dCTP, dGTP, TTP, pUTP (each 200 μM), 59 °C. Lane 0: no dNTPs/pUTP; lane +T: dATP, dCTP, dGTP, TTP; lane -T: dATP, dGTP, dCTP, lanes pU: dATP, dGTP, dCTP, pUTP.

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pGTP and pCTP, two new templates (3 and 4) were designed with single incorporation sites for the modified nucleotides at position 27. After 16 h, complete primer extension was observed in all four sets of experiments (Figure 3). In three out of four cases (incorporation of pUTP, pATP, and pGTP), the elongation of the primer was strongly slowed down after nucleotide 27 or 31, respectively. This effect is not observed for misincorporation (Figure S3) and indicates therefore a successful conversion of pUTP, pATP, and pGTP with template 2 or 3, respectively. In the presence of pCTP and template 4, the full-length product is already reached after 1 h so that misincorporation is more probable compared to incorporation of the 2′-modified cytidine (pCTP). Furthermore, in the case of the primer extension with pGTP and template 3, a double band is observed which indicates a mixture of propargyl-modified and unmodified DNA.

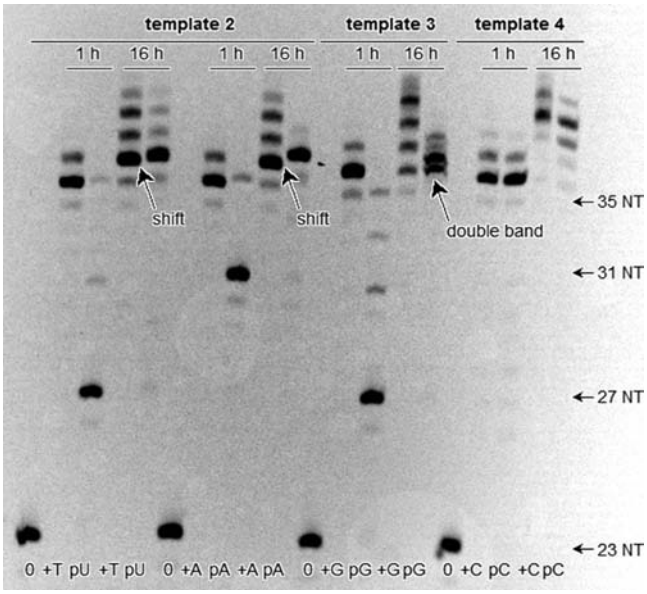

Figure 3. PAGE of running start primer extension with Therm III and templates 2−4: Primer (750 nM), template (900 nM), Therm III (1.0 U), dNTPs/pNTPs (200 μM), 60 °C. Lane 0: no dNTPs/pNTPs, lanes +T, +A, +G, +C: dATP, dCTP, dGTP, TTP, lane pU: dATP, dCTP, dGTP, pUTP, lane pA: dCTP, dGTP, TTP, pATP, lane pG: dCTP, dATP, TTP, pGTP, lane pC: dATP, dGTP, TTP, pCTP.

Finally, the modification of oligonucleotides was tested in a "click"-type reaction with nile red azide **5**[22,23] (Figure 4). The reaction volumes for the previous primer extension were enhanced such that 1.0 nmol modified oligonucleotides was yielded in each experiment. The enzymatic reaction was quenched with a phenolic solution and the modified oligonucleotide was purified by HPLC. The cycloaddition was performed under published standard conditions.[18,29] This includes aq. DMSO/*t*-BuOH as solvent and excess of nile red azide **5**, Cu(I), TBTA ligand, and ascorbate. Aliquots were taken after 5, 15, and 30 min, quenched by addition of EDTA and analyzed by PAGE. Gel imaging was performed at two different wavelengths (Figure 4). The green emission shows all primer-extended oligonucleotides that are present in the sample due to the presence of fluorescein as covalent label. As mentioned above, the fact that two or more bands are observable in the gel images can be assigned to template-free, further elongation.[28] Only oligonucleotides that were both correctly extended and successfully modified by the "click"-type cycloaddition should exhibit an additional red emission of the nile red label. As expected, only green emission is observed at the beginning (0 min) of the reaction in all four samples since the nile red label is present but not yet covalently attached to the oligonucleotides. With increasing reaction time, new gel bands with red emission appear in the samples with oligonucleotides bearing 2′-*O*-propargylated U, A, and G, but not with C. This observation verifies the successful incorporation and modification of the first three modified pNTPs. On the other hand, the latter result supports the previous doubts and indications that incorporation of pCTP failed.

In conclusion, we show that the 2′-*O*-propargylated nucleosides (of U, A, G, and C) can be converted efficiently to the corresponding pNTPs **1**−**4**. Primer extension experiments revealed that these triphosphates (except pCTP), although they represent RNA-type monomers, can be successfully incorporated in oligonucleotides using the so-called Therminator DNA polymerases. Most importantly, ethynyl groups as single modifications of the enzymatically extended oligonucleotides

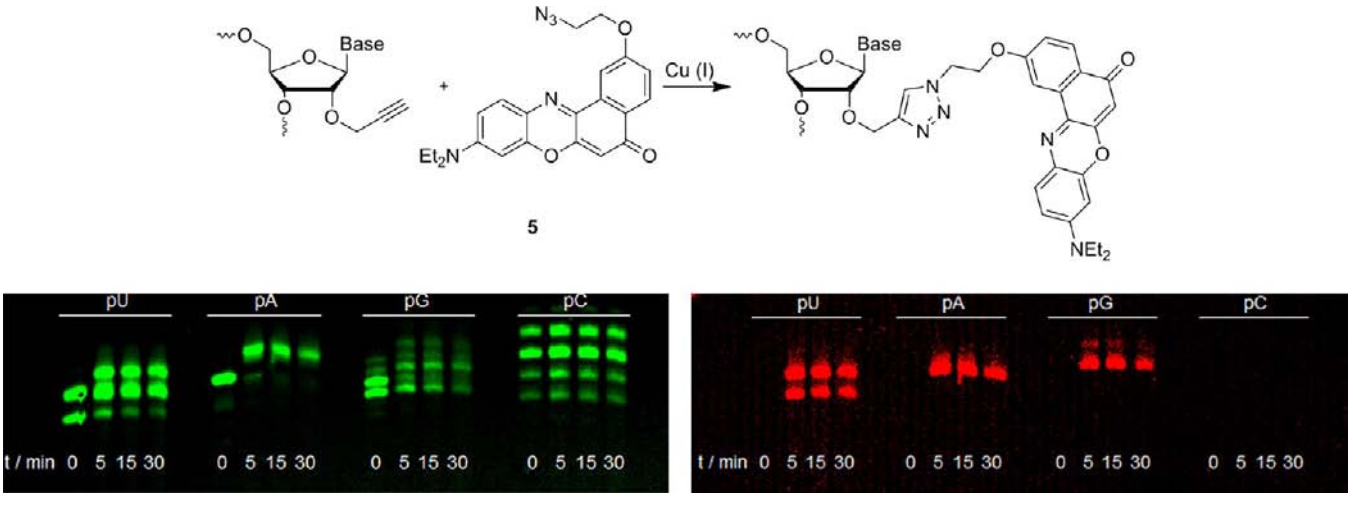

Figure 4. PAGE to analyze postsynthetic CuAAC of 2′-*O*-propargyl-modified DNA and nile red azid **5**: left, green fluorescence of fluorescein; right, red fluorescence of nile red.

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can be applied in postsynthetic labeling which was representatively shown by the "click"-type cycloaddition with the fluorescent nile red azide **5**. These results show that the 2′-position, as one of the most important modification site in antisense and other oligonucleotides, is now accessible for enzymatic DNApreparation. In particular, pUTP, pATP, and pGTP have the potential to be used in PCR amplification and subsequent bioorthogonal labeling methodologies.

■ ASSOCIATED CONTENT

Supporting Information

Description of the synthesis of the pNTPs **1**−**4**, images of NMR spectra and MS analysis, and images of additional PAGE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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