JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

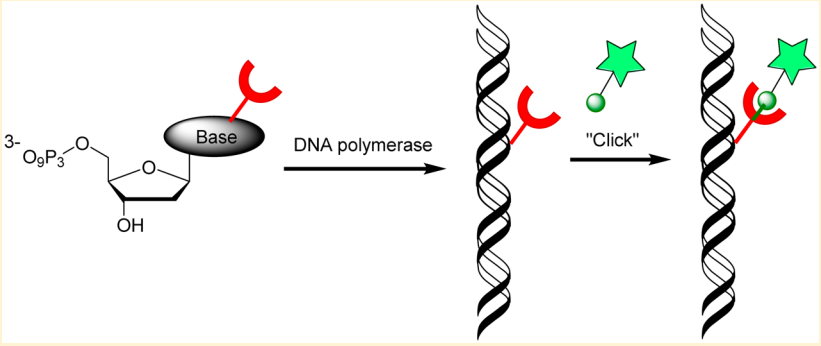
Scope and Limitations of Typical Copper-Free Bioorthogonal Reactions with DNA: Reactive 2′-Deoxyuridine Triphosphates for Postsynthetic Labeling

Marcus Merkel,[†] Stefanie Arndt,[†] Damian Ploschik,[†] Gergely B. Cserép,[‡] Ulrike Wenge,[†] Péter Kele,[‡] and Hans-Achim Wagenknecht*[,†]

[†]Institute of Organic Chemistry, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany

[‡]Chemical Biology Research Group, Institute of Organic Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar tudósok krt. 2, H-1117 Budapest, Hungary

Supporting Information



ABSTRACT: Four triphosphates of 2′-deoxyuridine that carried the following bioorthogonally reactive groups were synthesized by organic−chemical methods. Two triphosphates with tetrazines and one with a cyclopropene moiety were designed for Diels−Alder reactions with inverse electron demand, and one triphosphate with a tetrazole core was designed for the "photoclick" cycloaddition. These triphosphates were not only successfully applied for oligonucleotide preparation by standard DNA polymerases, including Hemo KlenTaq, Vent, and Deep Vent, but also bypassed for full length primer extension products. Fluorescent labeling of the primer extension products was achieved by fluorophores with reactive counterparts and analyzed by polyacrylamide gel electrophoresis mobility shifts. The tetrazine−oligonucleotide conjugates were reacted with carboxymethylmonobenzocyclooctyne- and bicyclononyne-modified fluorophores. The yield of these postsynthetic reactions could significantly be improved by a more stable but still reactive nicotinic acid-derived tetrazine and by changing the key experimental conditions, mainly the pH of 7.2 and the temperature of 45−55 °C. The cyclopropene−oligonucleotide conjugate could be successfully labeled with a tetrazine-modified rhodamine in very good yields. The "photoclick" cycloaddition between tetrazole−oligonucleotide conjugates and a maleimide-modified dye worked quantitatively. The combination of primer extension, bypass, and bioorthogonal modification works also for double and triple labeling using the cyclopropene-modified 2′-deoxyuridine triphosphate.

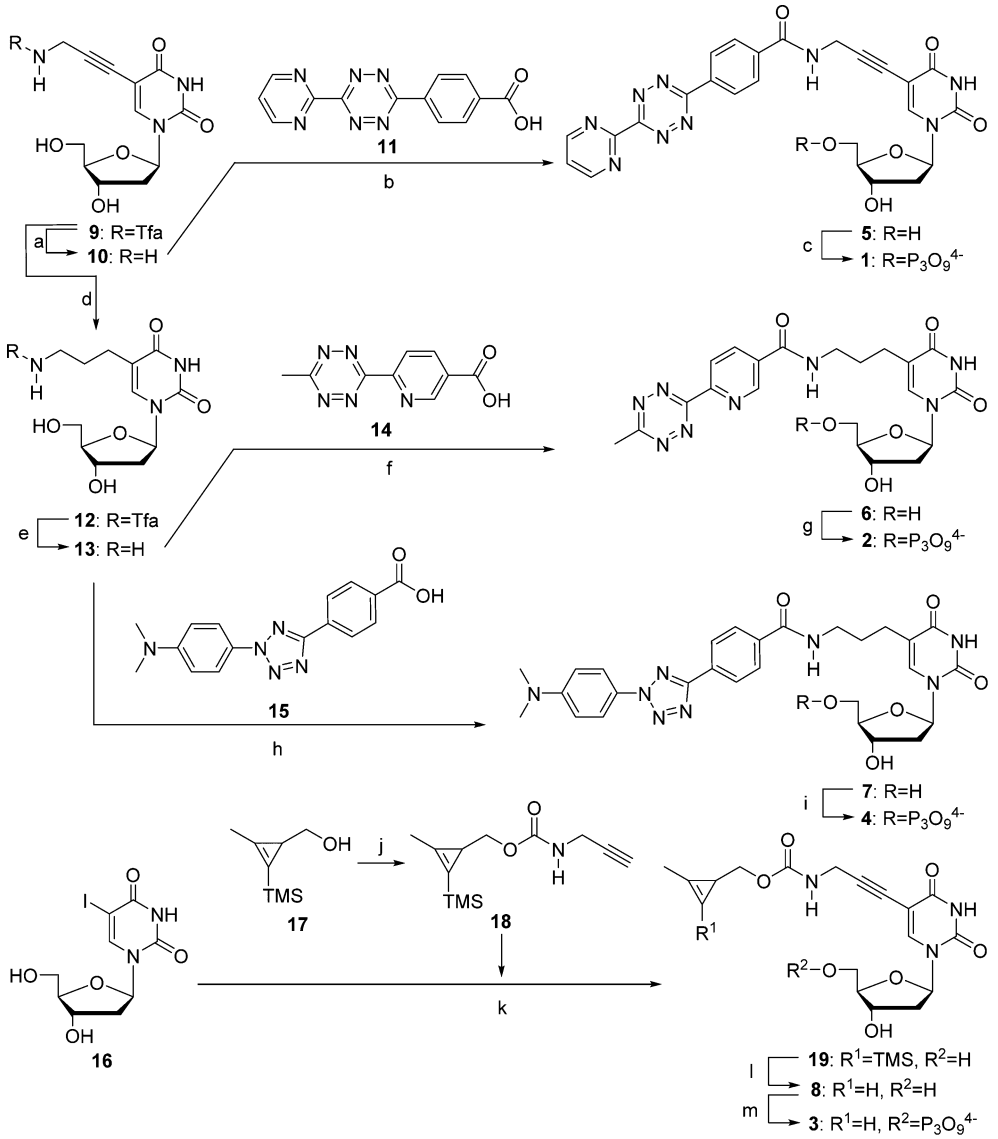
INTRODUCTION

Bioorthogonal modifications of biopolymers with fluorescent probes considerably facilitate the understanding of cellular processes, e.g., glycosylation of cell membranes.[1,2] Imaging biological events at the molecular level, today called "molecular imaging",[3] offers unique insights into the functioning of biological systems in living cells. The chemistry for attachment of fluorescent labels to biomolecules in general,[4] and nucleic acids in particular,[5] is therefore of utmost importance in many applications of biological imaging. With respect to DNA and RNA, new strategies for bioconjugation with nucleic acids have been developed over the past decade. The broadly applied Cu(I)-catalyzed cycloaddition between acetylene-modified nucleic acids and azide-bearing labels is a well-established methodology.[6−9] There are also several reports on the synthetically more challenging introduction of azides into RNA[10,11] and DNA[12,13] that react with ethynyl-modified labels. Although copper catalysis *in vivo* is highly problematic, this "click"-type chemistry and other transition metal-mediated ligations have successfully been applied for proteins in living cells.[14,15] Copper ion impurities, however, have to be removed very carefully from modified nucleic acid modifications prior to intracellular transport because they exhibit significant cytotoxicity.[16] Hence, it is not surprising that alternatives were elucidated for efficient copper-free and transition metal-free

Received: May 20, 2016
Published: August 11, 2016

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

Scheme 1. Synthesis of Nucleosides 5−8 and Nucleoside Triphosphates 1−4[a]

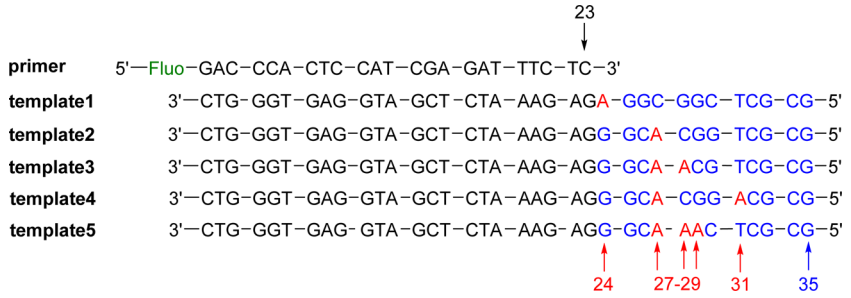


[a](a) NH_3, conc, aqueous solution, 17 h, rt, quant; (b) **11**, EDC, HOAt, $(iPr)_2NEt$, DMF, 19 h, rt, 70%; (c) (1) $POCl_3$, proton sponge, trimethylphosphate, 0 °C, 3 h; (2) $(nBu_3NH)_2P_2O_7$, nBu_3N, absolute (abs.) DMF, 15 min, 0 °C; (3) TEAB buffer, 40 min, 0 °C to rt, 3%; (d) $Pd(OH)_2$, $HSiEt_3$, MeOH, 24 h, rt, 79%; (e) NH_3, conc, aqueous solution, 17 h, rt, quant; (f) **14**, HBTU, HOBt, $(iPr)_2NEt$, abs. DMF, 24 h, rt, 49%; (g) (1) proton sponge, trimethylphosphate, $POCl_3$, 6 h, −5 °C; (2) $(nBu_3NH)_2H_2P_2O_7$, nBu_3N, 15 min, rt; (3) TEAB buffer, 40 min, rt, 16%; (h) **15**, HBTU, HOBt, $(iPr)_2NEt$, abs. DMF, 24 h, rt, 28%; (i) (1) proton sponge, trimethylphosphate, $POCl_3$, 6 h, −5 °C; (2) $(nBu_3NH)_2H_2P_2O_7$, nBu_3N, DMF, 15 min, rt; (3) 0.1 M TEAB buffer, 40 min, rt, 17%; (j) (1) carbonyldiimidazole, THF, 3 h, rt; (2) propargylamine, $(iPr)_2NEt$, overnight, rt, 98%; (k) **18**, $Pd(OAc)_2$, Et_3N, CuI, DMF, 24 h, rt, 35%; (l) 1 M TBAF in THF, DMF, overnight, rt, 99%; (m) (1) $POCl_3$, proton sponge, trimethylphosphate, −15 °C, 5 h; (2) $(nBu_3NH)_2H_2P_2O_7$, nBu_3N, abs. DMF, 15 min, rt; (3) TEAB buffer, 4 h, rt, 9%.

methods for nucleic acid modifications. The main alternatives are new DNA and RNA building blocks for strain-promoted azide−alkyne cycloadditions. We have recently summarized these reactions, including Diels−Alder reactions with normal and inverse electron demand, "photoclick" cycloadditions, oxime ligations, and thiole-ene additions as possible bioorthogonal transformations.[5] The implementation of these fundamentally different types of reactivities into nucleoside triphosphates for DNA polymerase-assisted modification of oligonucleotides has not yet been fully achieved. Mainly, nucleoside triphosphate building blocks with azide[13,17,18] for Staudinger-type ligations and copper-catalyzed azide−alkyne cycloadditions (CuAAC) with terminal acetylenes, with cyclooctynes[19] for strain-promoted cycloadditions with azides, with dienes[20] for Diels−Alder-type ligations, and with *trans*-cyclooctenes/vinyl groups[17,21,22] for inverse electron demand Diels−Alder-type (iEDDA) reactions were realized as bioorthogonally useful functionalities. This is disappointing because DNA polymerase-assisted preparation and modification have the advantage that it works under very mild conditions and allows the synthesis of longer, biologically relevant and also multilabeled oligonucleotides. To extend the bioorthogonal toolbox for nucleic acids, we report here the synthesis of four new 2′-deoxyuridine triphosphates that bear tetrazine, tetrazole, or cyclopropene moieties as bioorthogonally reactive groups at position 5. This site of attachment is typically tolerated by DNA polymerases according to recent results.[23] Those structural insights into the processing of nucleobases modified with

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

Scheme 2. Primer and Templates 1 (standing start) and 2−5 (running start) for Primer Extension Experiments



several different labels or groups showed that position 5 is generally well tolerated; however, the type of modification may impact the enzymatic efficiency. Incorporation of these modified building blocks into oligonucleotides was achieved during primer extension experiments to test the applicability and tolerance of different DNA polymerases together with the subsequent postsynthetic labeling potential with correspondingly functionalized fluorophores. These experiments revealed the scope and limitations of the iEDDA and "photoclick" reactions as two important bioorthogonal modifications.

RESULTS AND DISCUSSION

In principle, there are two metal-free, bioorthogonal reactions that are considered currently for nucleic acid modifications, i.e., the inverse electron demand Diels−Alder reaction of tetrazines and strained alkenes/alkynes and the light-induced cycloaddition of tetrazoles and alkenes ("photoclick"). The rationale behind using these reactions for biomolecular transformations is that their reaction rates are comparable to the rates of copper-catalyzed azide-acetylene cycloadditions (e.g., $k \sim 10-200\ M^{-1}\ s^{-1}$, $k \sim 1-10^4\ M^{-1}\ s^{-1}$, and $k \sim 60\ M^{-1}\ s^{-1}$ for CuAAC, iEDDA, and "photoclick" reactions, respectively).[24−29] Tetrazines represent a very delicate bioorthogonally reactive group with respect to nucleic acids because fast and efficient reactivity comes together with lability, especially in aqueous media. A phenyl group on one side of the tetrazine core and a pyrimidine moiety on the other side represent one of the best compromises between high reactivity and necessary stability[30] and were attached via the rigid aminopropynyl linker as part of triphosphate **1**. Recently, we described that the use of nicotinic acid is helpful with respect to the stability under physiological conditions,[31] and hence, we decided to apply it in combination with a methyl group at the other side of the tetrazine in triphosphate **2**. Furthermore, very recently, we found that a saturated alkyl and thus more flexible linker between the bioorthogonally reacting group and position 5 of the 2′-deoxyuridine improves the optical properties of the finally conjugated fluorescent label compared to those of the rigid aminopropynyl linker.[32] Hence, in the second triphosphate **2**, this advanced propyl linker was applied. Some of the most reactive partners for iEDDA reactions with tetrazines are cyclopropenes. To gain access to this reactive group for iEDDA with DNA, we designed triphosphate **3**. It bears again the rigid aminopropynyl linker because the cyclopropene moiety was not stable under the hydrogenation conditions that were applied to reduce this linker to the saturated alkyl chain. With respect to the tetrazole chemistry, our group was the first to present a tetrazole phosphoramidite derivative as a DNA building block suitable for solid-state DNA synthesis and subsequent "photoclick" ligation with a maleimide dye upon excitation by LEDs at 365 nm, which falls outside of the nucleic acid absorption window.[33] This push−pull-substituted diaryltetrazole unit was incorporated into triphosphate **4** using the flexible alkyl linker.

The syntheses of unphosphorylated nucleosides **5**−**7** [except **8** (Scheme 1)] started from trifluoroacetyl-protected 5-(3″-aminopropynyl)-2′-deoxyuridine **9** that can be obtained in a few steps from commercially available 5-iodo-2′-deoxyuridine following literature procedures.[34] Deprotection by aq NH_3 at room temperature furnished precursor **10** for nucleoside **5** in 88% yield. The diaryltetrazine unit **11** was synthesized according to the literature method[35,36] and reacted with **10** in the presence of EDC/HOAt to obtain nucleoside **5** in 70% yield.

The key step for converting the aminopropynyl linker to the respective alkyl chain in nucleosides **6** and **7** was the hydrogenation by treatment with $HSiEt_3$ using 20% $Pd(OH)_2/C$ as a catalyst.[37] This reaction gave the protected nucleoside precursor **12** in up to 79% yield. The TFA protecting group of **12** was then removed to yield amine **13**, which is the common precursor for nucleosides **6** and **7**. The nicotinic acid-derived tetrazine **14**[31] was attached to **13** using HBTU/HOBt to give nucleoside **6** in 49% yield. Likewise, the diaryltetrazole moiety **15** that was obtained according to the literature[33] was coupled to **13** with HBTU/HOBt to obtain nucleoside **7** in 28% yield.

The synthesis of the cyclopropene-modified nucleoside **8** had to follow a different route because the cyclopropene group was not stable enough for the typical syntheses mentioned above. The TMS-protected cyclopropene **17**[38] was first treated with carbonyldiimidazole and then coupled to propargylamine by a urethane linker. Subsequently, the Sonogashira-type coupling to 5-iodo-2′-deoxyuridine **16** gave the TMS-protected nucleoside **19** in moderate yield (35%). This TMS group could easily be removed by treatment with TBAF and furnished nucleoside **8** in quantitative yield.

The unprotected nucleosides **5**−**8** were directly converted to the corresponding triphosphates using the methodology of Ludwig et al.[39] Accordingly, nucleoside **5** was phosphorylated using $POCl_3$ in trimethylphosphate in the presence of a proton sponge, then converted to the cyclic triphosphate using nBu$_3$N and $(n\text{Bu}_3\text{NH})_2\text{P}_2\text{O}_7$ in DMF, and finally hydrolyzed with a TEAB buffer solution. Ion exchange chromatography followed by reversed phase C18 chromatography was performed, yielding triphosphate **1** in 3% yield. Triphosphates **2**−**4** were synthesized using the same reagents under slightly different conditions (see the Experimental Section). Purification of the latter two triphosphates required only reverse phase HPLC separation on a preparative C18 column. The 2′-deoxyuridine triphosphates **2** and **3** were obtained in yields of 16 and 17%, respectively, and the triphosphate **4** was obtained in 9% yield.

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

The primer extension setup (Scheme 2) follows our previous work:[40] the primer contains 23 nucleotides and carries a fluorescein label at the 5′-end to allow fluorescent detection after gel electrophoresis. Templates 1−5 are 35 nucleotides long and were designed for standing start and running start experiments. Templates 1 and 2 bear only one adenosine, either at position 24 (standing start) or at position 27 (running start), to selectively insert 2′-deoxyuridine triphosphates **1**−**4** at these distinct positions. Templates 3 and 4 should give double incorporation, either adjacently or separated by three nucleotides, and template 5 should yield a triple incorporation. Three different DNA polymerases were mainly tested for the primer extension experiments: Vent,[41,42] Deep Vent,[43] and Hemo KlenTaq polymerase.[44] All polymerases lack the 3′−5′-exonuclease activity.

In the case of the tetrazine-modified 2′-deoxyuridine triphosphate **1**, all three DNA polymerases tolerated the tetrazine moiety in running start experiments. Gel electrophoretic analysis of the primer extension products with template 2 (Figure 1) clearly revealed the full length

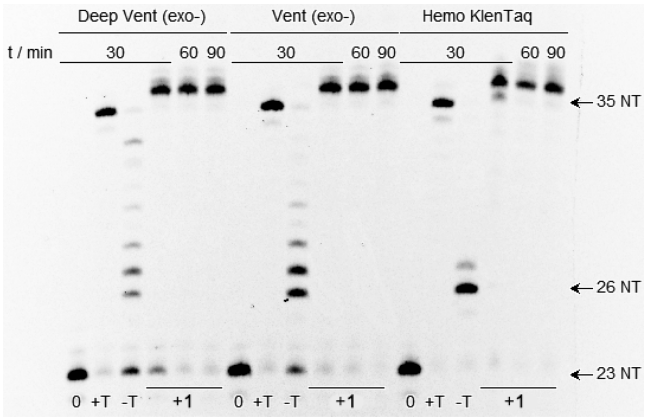


Figure 1. PAGE analysis of primer extension experiments with **1**. Primer (750 nM), template 2 (900 nM), polymerase (0.5 unit U), dNTPs, and **1** (100 μM), 72 °C for Deep Vent and Vent, 64 °C for Hemo KlenTaq: lanes 0, without dNTPs; lanes +T, with dATP, dGTP, dCTP, and TTP; lanes −T, with dATP, dGTP, and dCTP; lanes +**1**, dATP, dGTP, dCTP, and **1**.

oligonucleotides in all cases. Without **1** or TTP, only 26mer truncated extension products were obtained. Remarkably, there is a small shift in gel electrophoretic mobility between the tetrazine-modified 35mer oligonucleotide and the corresponding elongated unmodified oligomer (with TTP instead of **1**), which further supports successful incorporation of the modified triphosphate **1**. Only with Hemo KlenTaq polymerase was a small amount of misincorporation detectable according to the occurrence of a second and small band underneath the fully elongated product.

For the postsynthetic Diels−Alder reaction between the extended and tetrazine-modified oligonucleotide and the COMBO-modified rhodamine dye **20**,[45] the primer extension experiments were repeated with template 2 on a semi-preparative scale of 9.5 μM (1 nmol). Both reacting partners were dissolved in a 20:1 DMSO/water mixture and incubated for 30 min. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis (Figure 2) revealed an additional band with slower mobility that exhibits fluorescence not only in the green range (excitation by LED at 470 nm, emission at 535 nm) but more importantly also in the red range (excitation by LED at 540 nm, emission at 605 nm). Although it is not possible to exactly quantify the yield of this postsynthetic labeling step, the gel bands indicate a very low yield (5−10%). Extension of reaction times and addition of more COMBO-modified rhodamine **20** did not improve the yield. Hence, it is likely that the stability of this tetrazine moiety is not sufficient to quantitatively "survive" the conditions of primer extension.

As we recently demonstrated with nicotinic acid-derived tetrazines, the reactivity for inverse electron demand Diels−Alder reactions is strongly affected by the electronic features and steric demand of the substituents on the tetrazine framework.[31] To reach a compromise between reactivity and stability and thereby aim to improve the postsynthetic modification yield, we aimed to incorporate the methyl- and nicotinic acid-substituted tetrazine **14** into DNA for subsequent tagging with fluorophores.[31] In the aforementioned study in which a phosphoramidite of nucleoside **6** was embedded into oligonucleotides using solid phase oligonucleotide synthesis. Because of the general instability of tetrazine moieties under harsh basic conditions used for DNA cleavage from the solid support, the postsynthetic modification with the COMBO-modified dye as a dienophile had to be performed with the DNA on the support (on bead). Thereby, the labile tetrazine converts the more stable diazine before the DNA is cleaved from the support. Hence, triphosphate **2** represents an important alternative because it carries the same tetrazine motif as the reported phosphoramidite but allows enzymatic incorporation. The modification at position 5 of 2′-deoxyuridine of triphosphate **2** was tolerated by conventional polymerases such as Deep Vent and Hemo KlenTaq both in standing start experiments with template 1 (see the Supporting Information) and in running start experiments with template 2 (Figure 3). However, the typically applied conditions for primer extension experiments, especially pH and temperature, were still rather harsh for tetrazines. Because of the low yield of the tetrazine-induced rhodamine conjugate in the previous example with triphosphate **1** (see above), the pH for all experiments was set to neutral (7.2) and the temperature was also lowered. These experimental changes required an additional polymerase screening that showed full length extension products within 30 min at 45−55 °C only with KOD XL polymerase, while Deep Vent and Hemo KlenTaq required higher temperatures (Figure 3).

After successful primer extension with KOD polymerase, the full length oligonucleotide product-containing solution was desalted, concentrated, and dissolved in water to reach a final concentration of 10 μM. Ligation steps with either COMBO (**20**) or BCN (**21**) containing dyes were performed in a 1:1 DMSO/water mixture for up to 120 min. It is evident from the corresponding gel images (Figure 4) that the yields of dye modification with triphosphate **2** after primer extension were significantly improved compared to those of the previous attempt with the tetrazine triphosphate **1**. The determined yields ranged between approximately 20−25% (**20**) and 35−40% (**21**). However, it was still not possible to postsynthetically label tetrazines in primer extension products by quantitative yields as might be expected for this type of bioorthogonal reaction. This problem must be assigned to the chemical lability of the tetrazine moiety because standing start primer extension with triphosphate **2** revealed similar yields after 30 min in comparison to those with the natural nucleoside triphosphates.

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

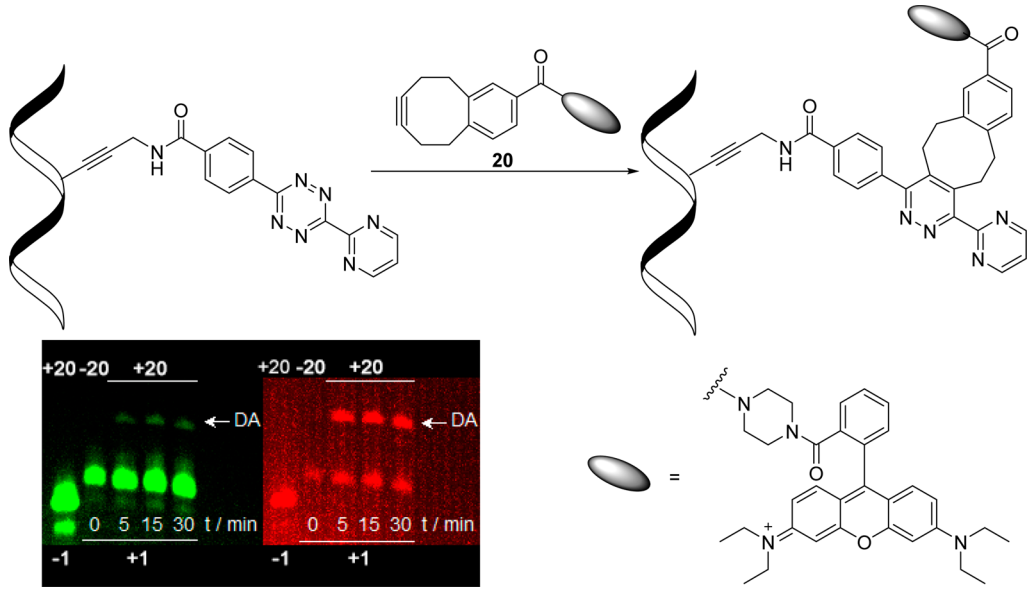


Figure 2. Reaction scheme and PAGE analysis (inset) of postsynthetic modification of the primer extension product by COMBO-modified rhodamine **20**. PAGE: lanes **−1**, primer extension product with dATP, dGTP, dCTP, and TTP, reacted with **20**; lanes **+1**, primer extension product with dATP, dGTP, dCTP, and **1**, reacted with **20**. DA marks the Diels−Alder product. Green indicates the fluorescence of fluorescein (λ_{exc} = 470 ± 20 nm, and λ_{em} = 535 ± 20 nm); red indicates the fluorescence of rhodamine ("clicked" **20**; λ_{exc} = 540 ± 10 nm, and λ_{em} = 605 ± 10 nm).

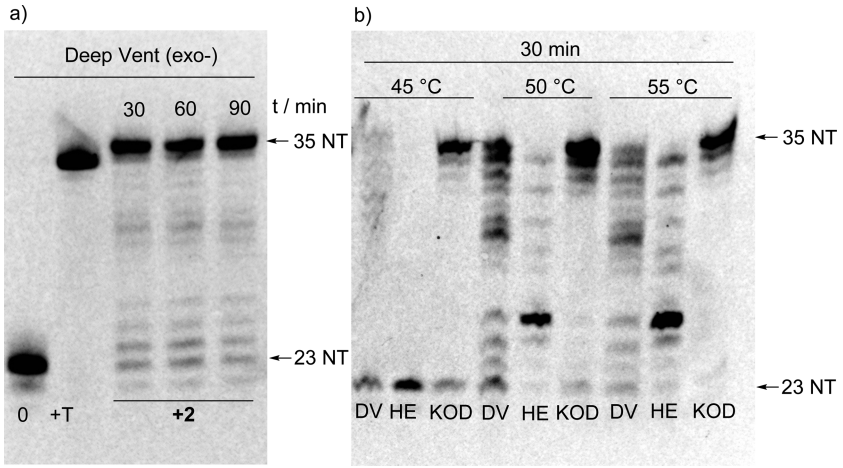


Figure 3. PAGE analysis of primer extension experiments with **2**. (a) Primer (750 nM), template 2 (900 nM), polymerase (1.0 unit), dNTPs, and **2** (200 μM), 72 °C for Deep Vent: lane 0, without dNTPs; lane +T, with dATP, dGTP, dCTP, and TTP; lanes +**2**, dATP, dGTP, dCTP, and **2**. (b) Primer (750 nM), template 2 (900 nM), dATP, dGTP, dCTP, and **2** (200 μM): lanes DV, Deep Vent used as polymerase (1.0 unit); lanes HE, Hemo KlenTaq used as polymerase (0.5 μL); lanes KOD, KOD XL used as polymerase (0.5 μL).

Generally, these results clearly give the limitation of this type of bioorthogonal reaction with respect to nucleic acid modification. Tetrazines not only fail in phosphoramidites as chemical DNA building blocks but also do not perform completely convincely in corresponding nucleoside triphosphates for biochemical DNA preparation.

For further acceleration of iEDDA reactions, the HOMO energy of the dienophile can be increased by the introduction of ring strain such as in cyclopropenes.[46] Devaraj et al. synthesized 1-methylcyclopropenes linked by amide or carbamate groups that showed remarkable second-order rate constants with tetrazines[47] and that we applied as a reactive group in nucleoside triphosphate **3**. It is important to mention that triphosphate **3** was prepared without the TMS group at the cyclopropene. All attempts to deprotect primer extension products that were obtained with a corresponding TMS-protected nucleoside triphosphate failed [with TBAF and HF·NEt_3 at up to 65 °C over up to 24 h (data not shown)]. As a result, the TMS-protected primer extension product could not be labeled by the tetrazine-modified dye **22** at all. As seen for **2**, standing start experiments with template 1 showed incorporation of **3** into the primer by Hemo KlenTaq, Vent, and Deep Vent polymerases (see the Supporting Information). Running start experiments with template 2 and the same polymerases bypassed the modification site and extended the primer to its full length within 30 min at 37 °C (Figure 5).

After successful primer extension with Deep Vent or Hemo KlenTaq polymerase, the full length oligonucleotide product was desalted, concentrated, and dissolved in water to reach a final concentration of 750 nM. Ligation with tetrazine-modified rhodamine **22** was performed in a 20:1 water/DMSO mixture for up to 90 min and showed successful labeling by an

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

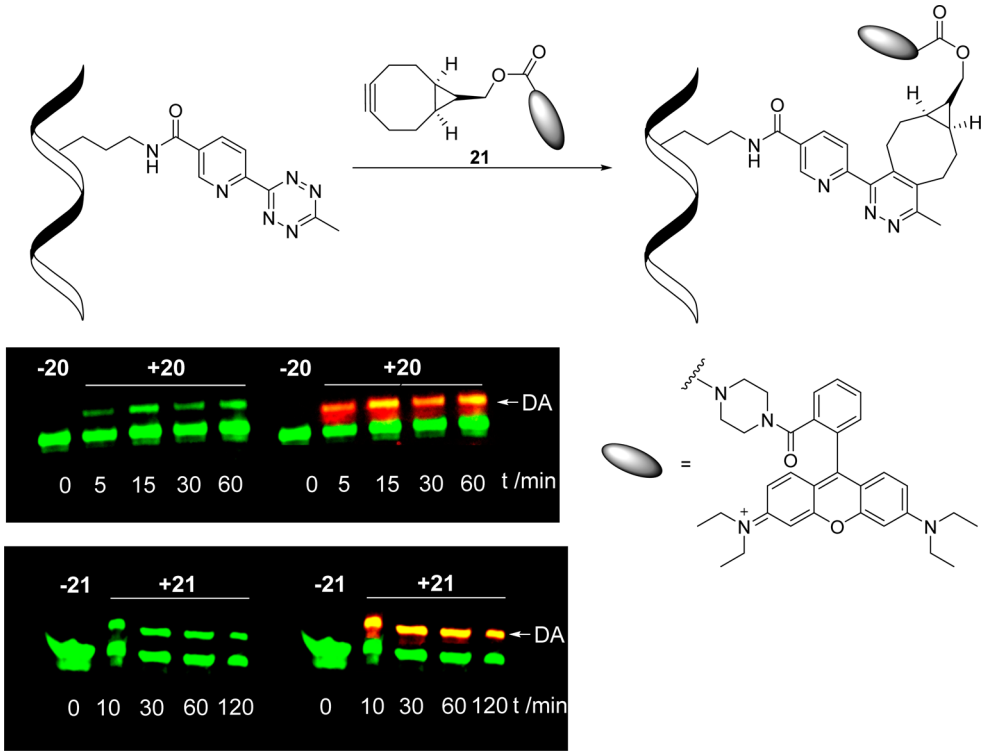


Figure 4. Reaction scheme and PAGE analysis (inset) of postsynthetic modification of the primer extension product with template 2 by COMBO-**20** and BCN-modified rhodamine **21**. PAGE: primer extension product with dATP, dGTP, dCTP, and **2**, reacted with **20** and **21**. DA marks the iEDDA product. Green indicates the fluorescence of fluorescein (λ_{exc} = 470 ± 20 nm, and λ_{em} = 535 ± 20 nm); red indicates the fluorescence of rhodamine ("clicked" **21**; λ_{exc} = 540 ± 10 nm, and λ_{em} = 605 ± 10 nm).

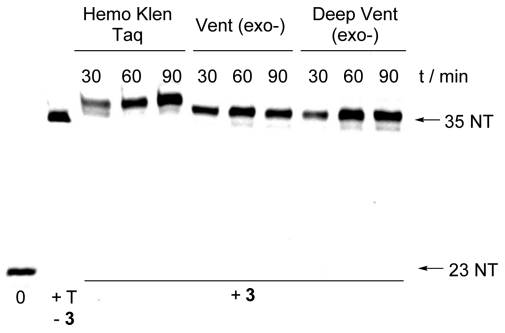


Figure 5. PAGE analysis of primer extension experiments with **3**. Primer (750 nM), template 2 (900 nM), polymerase [Hemo KlenTaq (0.5 μL), Vent (exo-) (1.0 unit), or Deep Vent (exo-) (1.0 unit)], dNTPs, and **3** (200 μM), 37 °C, at different extension times: lane 0, without dNTPs; lane +T, with dATP, dGTP, dCTP, and dTTP; lanes +**3**, dATP, dGTP, dCTP, and **3**.

additional slower-moving gel band (Figure 6). The yield of the labeling was approximately 80%, which is much better than those of the iEDDA reactions with tetrazine-modified oligonucleotides (see above). The reaction between the cyclopropene-modified oligonucleotide and tetrazine **22** gives regioisomers as products, as mentioned, for instance, by Devaraj, Houk, and co-workers.[48] The two different regioisomers are also enantiomeric mixtures (or diastereomeric mixture if the chirality of DNA is included), presumably racemic.

The "photoclick" reaction, which is the light-induced cycloaddition between diaryltetrazoles and activated alkenes, represents an important alternative to the previously described Diels–Alder reactions with inverse electron demand because it combines the reaction rate and specificity of a bioorthogonal reaction with the advantages of a photochemical process, mainly the spatial and temporal control.[25] The corresponding light-induced diarylnitrylimine represents a highly reactive 1,3-dipolar intermediate.[49] With respect to nucleic acid modification, this bioorthogonal reaction can be performed in water but has the intrinsic problem that typical 2,5-diphenyltetrazoles require a wavelength range that strongly overlaps with nucleic acid absorption. The combination of electron-donating and -withdrawing substituents at the phenyl groups solves this problem and allows excitation at 365 nm[50] by LEDs as an efficient and cheap light source, and thereby the nucleic acid modification as previously demonstrated by the chemical DNA synthesis.[33]

The primer extension experiments performed with triphosphate **4** revealed that the tetrazole modification at position 5 is perfectly tolerated by the applied DNA polymerases and allows incorporation within 30 min according to standing start experiments (see the Supporting Information). In the case of the successful running start experiments with template 2, there is a small upward shift of the primer extension product that is formed in the presence of **4** compared to the full length products with the four natural nucleoside triphosphates, indicating that the strand is slightly bulkier and the tetrazole moiety of **4** was successfully incorporated (Figure 7). Tetrazoles are typically much more stable than tetrazines, and no temperature problems were expected. With regard to the potential incorporation in bacterial or mammalian cells, however, it was demonstrated that successful incorporation of **4** can also be achieved at 37 °C using Hemo KlenTaq polymerase.

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527–7538

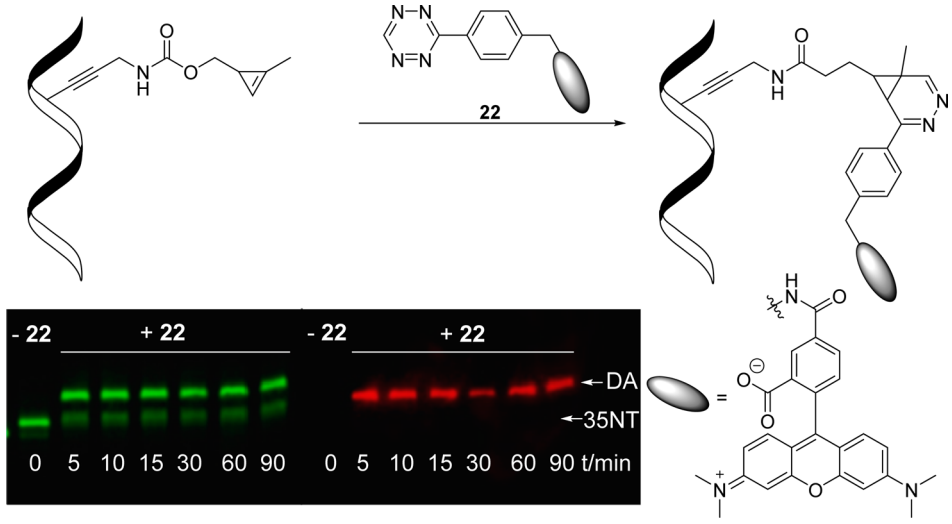


Figure 6. Reaction scheme and PAGE analysis (inset) of postsynthetic modification of the primer extension product with template 2 by tetrazine-modified rhodamine **22**. PAGE: primer extension product with dATP, dGTP, dCTP, and **3**, at 37 °C, reacted with **22**. DA marks the iEDDA product. Green indicates the fluorescence of fluorescein (λ_{exc} = 470 ± 20 nm, and λ_{em} = 535 ± 20 nm); red indicates the fluorescence of rhodamine ("clicked" **22**; λ_{exc} = 540 ± 10 nm, and λ_{em} = 605 ± 10 nm). Only one regiosomer of the bioconjugation product is depicted for the sake of clarity.

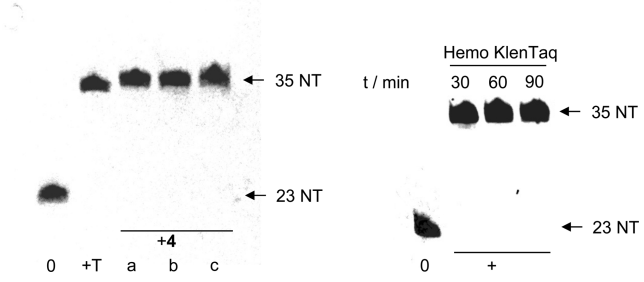


Figure 7. PAGE analysis of primer extension experiments of **4** with primer (750 nM), template 2 (900 nM), polymerase (0.5 unit), dNTPs (−dTTP) (100 μM), and **4** (120 μM). For the left panel, the extension time was 30 min for all lanes. Lane 0 refers to pure primer strand, and lane +T shows the complete product with all four natural dNTPs compared to the three remaining lanes, which show the full strand with dTetTP using the three different polymerases: (a) Deep Vent, (b) Vent, and (c) Hemo KlenTaq. The right panels shows PAGE analysis of primer extension using Hemo KlenTaq polymerase at 37 °C.

SDS−PAGE analysis of the subsequent "photoclick" reaction of the primer extension product with a 13-fold excess of the sulfo-Cy3 labeling reagent **23** (Figure 8) under irradiation for 30 min by 365 nm light from an LED revealed quantitative labeling. The "photoclick" reaction with maleimide **23** does not yield any regioisomeric mixture. The product is an enantiomeric mixture (or diastereomeric mixture if the chirality of DNA is included), presumably racemic.

For the iEDDA reaction with cyclopropene-modified DNA, the combination of primer extension, bypass, and bioorthogonal labeling was tested with templates 3−5 that should yield two and three modifications by providing up to three 2′-deoxyadenosines as insertion sites. Running start experiments with triphosphate **3** and Hemo KlenTaq and Deep Vent (-exo) polymerases showed complete primer extension after reaction for 30−60 min (see the Supporting Information). Postsynthetic labeling was again performed with the tetrazine-modified rhodamine **22** as the reactive counterpart to the cyclopropene moiety. The primer extension product with template 3 could be labeled with an approximately 50% yield at both adjacent nucleotides (Figure 9). The yield of doubly labeled oligonucleotide from the primer extension with template 4, where the reactive nucleotides are separated by 3 bp, is slightly higher, approximately 66%. Most remarkably, the primer extension product with template 5 that bears three modification sites adjacent to each other could by labeled at all three sites in approximately 70% yield. These results show that this bioorthogonal labeling approach can be successfully applied for up to at least three modification sites. Most importantly, (i) the altered PAGE mobilities of the dye-labeled oligonucleotide products compared to those of the unlabeled primer extension products and (ii) the fact that the initial band of the primer extension product vanishes during the labeling reaction nearly completely show that there are only very small amounts of misincorporation opposite the 2′-deoxyadenosine sites during the primer extension.

CONCLUSIONS

Postsynthetic modifications of DNA (and potentially also RNA) by copper-free bioorthogonal reactions represent an important method of choice for the introduction of fluorescent probes into oligonucleotides. In principle, this works for both the synthetic−chemical (by phosphoramidites) and the enzymatic−biochemical (by nucleoside triphosphates) preparation of nucleic acids. We focused here on the application of iEDDA and "photoclick" cycloaddition reactions as two typical, copper-free bioorthogonal reactions that work with second-order rate constants that are comparable or even faster than that of the broadly applied copper(I)-catalyzed cycloaddition between azides and acetylenes. Accordingly, primer extension and fluorophore labeling were studied with four nucleoside triphosphates that carried different reactive groups. The triphosphates **1**−**3** were designed for iEDDA reactions and triphosphate **4** for the "photoclick" cycloaddition. These triphosphates were synthesized as derivatives of 2′-deoxyuridines by organic−chemical methods and carried the reactive group at position 5 because DNA polymerases typically accept modifications at this position.[23] Remarkably, all triphosphates **1**−**4** were successfully applied for oligonucleotide synthesis with templates 1 and 2 by standard DNA polymerases,

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

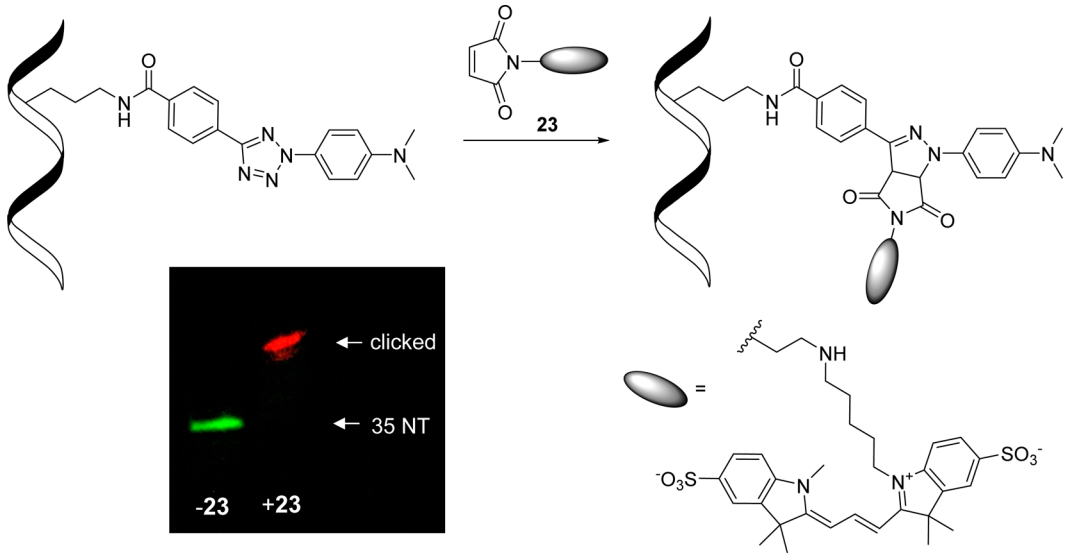


Figure 8. Reaction scheme and PAGE analysis (inset) of postsynthetic modification of the primer extension product with template 2 by maleimide-modified sulfo-Cy3 **23**;. PAGE: lane **−23**, primer extension product with dATP, dGTP, dCTP, and **4**, at 37 °C; lane **+23**, reacted with **23**. clicked marks the "photoclick" product. Green indicates the fluorescence of fluorescein (λ_{exc} = 470 ± 20 nm, and λ_{em} = 535 ± 20 nm); red indicates the fluorescence of sulfo-Cy3 ("clicked" **23**; λ_{exc} = 540 ± 10 nm, and λ_{em} = 605 ± 10 nm).

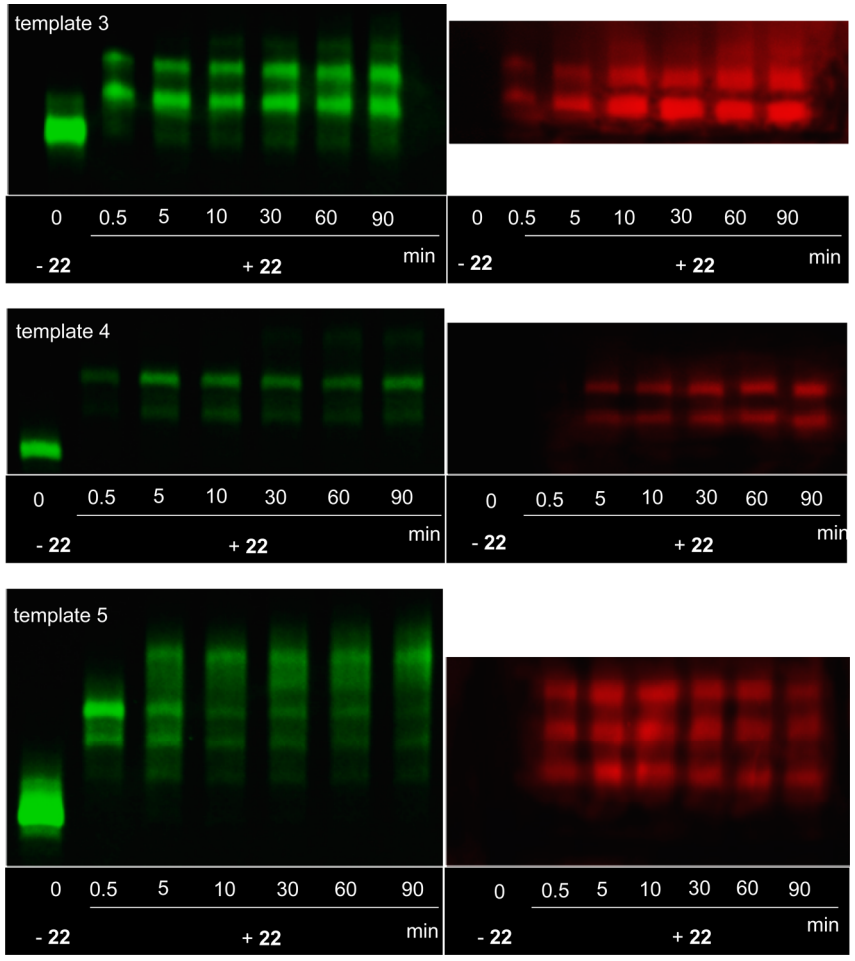


Figure 9. PAGE analysis of postsynthetic modifications of the primer extension products with templates 3 (top), 4 (middle), and 5 (bottom), Deep Vent (exo-) polymerase, by tetrazine-modified rhodamine **22**. Green indicates the fluorescence of fluorescein (λ_{exc} = 470 ± 20 nm, and λ_{em} = 535 ± 20 nm); red indicates the fluorescence of rhodamine ("clicked" **22**; λ_{exc} = 540 ± 10 nm, and λ_{em} = 605 ± 10 nm).

including Hemo KlenTaq, KOD XL, Vent, and Deep Vent. Standing start experiments revealed a selective incorporation of the modified 2′-deoxyuridine triphosphates **1**−**4** opposite to 2′-deoxyadenosine in template 1. With template 2, the

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

triphosphates **1**–**4** were not only incorporated once at the desired sites but also bypassed to full length primer extension products. Fluorescent labeling of the primer extension products was achieved by fluorophores with reactive counterparts and analyzed by PAGE mobility shifts and dual fluorescence readout. The tetrazines of oligonucleotides prepared with triphosphates **1** and **2** were reacted with COMBO- and bicyclononyne-modified fluorophores **20** and **21**. The yield of this iEDDA reactions could significantly be improved by a more stable but still reactive nicotinic acid-derived tetrazine (according to previous studies,[31] in triphosphate **2**), and by changing the key experimental conditions, mainly, the neutral pH (7.2) and the comparably low temperature (45–55 °C, especially with KOD XL polymerase). The cyclopropene of oligonucleotides that were prepared with triphosphate **3** could be successfully labeled with the tetrazine-modified rhodamine **22** but only if the TMS group was removed from the cyclopropene before the triphosphate synthesis. The "photo-click" cycloaddition between oligonucleotides prepared with **4** and maleimide-modified dye **23** proceeded with a quantitative yield. Representatively, triphosphate **3** was additionally tested with templates 3–5 that direct the primer extension to incorporate two or even three cyclopropene-modified nucleosides. The fluorescent labeling with the tetrazine-modified rhodamine **22** was successful; however, quantitative labeling could not be achieved.

In principle, the important advantage of this approach is that it is necessary only to chemically develop single-nucleoside triphosphates bearing a chosen reactive functional group that can be applied to a variety of different fluorophores. Chemoselectivity and bioorthogonality are provided by these postsynthetic labeling methods and should allow the performance of this type chemistry also in living cells (as recently shown for the strain-promoted cycloaddition)[32] or even living organisms, such as zebrafish. Hence, the scope of these reactions is, in principle, broad. However, the limitation is set by the lability of the bioorthogonally reactive groups under primer extension conditions, which calls into question their stability under physiological conditions, too. Not everything that successfully works for proteins can be accomplished with nucleic acids. According to the results presented herein, the tetrazines are especially problematic in phosphoramidites as chemical DNA building blocks (except modifications on solid phase)[31] and also unexpectedly labile in nucleoside triphosphates and during biochemical DNA preparation.

EXPERIMENTAL SECTION

Materials and Methods. Chemicals and dry solvents were purchased and used without further purification unless otherwise mentioned. Templates and primers were commercially obtained. TLC was performed on 0.20 nm silica gel 60 with a 254 nm indicator. Flash chromatography was performed with silica gel 60 (60–43 μm). NMR spectra were recorded on 300, 400, and 500 MHz spectrometers in deuterated solvents (^{1}H at 300, 400, or 500 MHz, ^{13}C at 75, 100, or 125 MHz, and ^{31}P at 101 MHz). Mass spectrometry (MALDI) was performed in linear negative mode [the matrix for DNA being a 2:1 mixture of 2,4,6-trihydroxyacetophenone (0.3 M in ethanol) and diammonium citrate (0.1 M in H_2O)].

Synthesis of 5-(3-Aminopropynyl)-2′-deoxyuridine (10). The preparation was performed according to the literature method.[34] The spectroscopic data were in agreement with the literature.

Synthesis of 5-{4-[6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl]-benzoyl}uridine (5). In a dry, argon-flushed flask, **10** (50 mg, 0.2 mmol, 1.0 equiv) was dissolved in abs. DMF (2 mL). EDC (38 μL, 30 mg, 0.2 mmol, 1.2 equiv), HOAt (0.21 mL of a 1.0 M solution in abs. DMF, 0.2 mmol, 1.2 equiv), $(i\mathrm{Pr})_2$NEt (61 μL, 50 mg, 0.4 mmol, 2.0 equiv), and **11**[35,36] (100 mg, 0.3 mmol, 1.5 equiv) were added. After the reaction mixture had been stirred at room temperature for 19 h, the reaction was quenched by adding MeOH (10 mL). The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (10:1 CH_2Cl_2/MeOH). The purple and solid product were obtained in 70% yield (70 mg, 0.1 mmol). R_f (8:1 CH_2Cl_2/MeOH) = 0.3. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.12 (t, J = 6.5 Hz, 2 H, 2′-H), 3.49–3.69 (m, 2 H, 5′-H), 3.80 (q, J = 3.3 Hz, 1 H, 3′-H), 4.22 (q, J = 4.1 Hz, 1 H, 4′-H), 4.36 (d, J = 5.5 Hz, 2 H, CH_2), 5.12 (t, J = 5.0 Hz, 1 H, 5′-OH), 5.25 (d, J = 4.2 Hz, 1 H, 3′-OH), 6.12 (t, J = 6.7 Hz, 1 H, H-1′), 7.85 (t, J = 4.9 Hz, 1 H, Ar-H), 8.15–8.26 (m, 3 H, Ar-H, H-6), 8.69 (d, J = 8.2 Hz, 2 H, Ar-H), 9.21 (d, J = 4.9 Hz, 2 H, Ar-H), 9.33 (t, J = 5.4 Hz, 1 H, N-H), 11.65 (s, 1 H, H-3). ^{13}C NMR (126 MHz, DMSO-d_6): δ 29.4, 45.6, 61.0, 70.2, 74.4, 84.7, 87.6, 89.5, 98.1, 123.0, 128.2, 128.4, 134.1, 137.5, 143.8, 149.4, 158.5, 159.1, 161.6, 162.9, 163.2, 165.1. HRMS (ESI): $[M - H]^-$ calcd for $C_{25}H_{20}N_9O_6^-$ m/z 542.1542, found m/z 542.1535.

Synthesis of 5-{4-[6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl]-benzoyl}uridine 5′-Triphosphate (1). In a dry, argon-flushed flask, **5** (20 mg, 0.04 mmol, 1.0 equiv) and proton sponge (12 mg, 0.05 mmol, 1.5 equiv) were dissolved in trimethylphosphate (0.7 mL) and cooled to 0 °C before $POCl_3$ (4.0 μL, 7 mg, 0.04 mmol, 1.2 equiv) was slowly added. After the mixture had been stirred at 0 °C for 1.5 h, proton sponge (0.5 equiv) and $POCl_3$ (0.5 equiv) were added. After the mixture had been stirred for a further 1.5 h, a solution of $n\mathrm{Bu}_3$N (43 μL, 34 mg, 0.18 mmol, 5.0 equiv) and $(n\mathrm{Bu}_3\mathrm{NH})_2\mathrm{P}_2\mathrm{O}_7$ (0.45 mL of a 0.4 M solution in DMF, 0.18 mmol, 5.0 equiv) was added. After the mixture had been stirred for 15 min, the reaction was quenched by adding TEAB buffer (2.0 mL, 0.1 M, pH 7.5). After the mixture had been stirred at 0 °C for 10 min and at room temperature for 30 min, the reaction was completed. The crude product was purified by ion exchange chromatography (DEAE Sephadex A-25) using a gradient (from 0.1 to 1.0 M TEAB buffer) and by reversed phase HPLC using TEAA (50 mM, pH 7) and MeCN as eluents. The solvent of the product-containing fractions was removed under reduced pressure, and the ammonium ions were exchanged with Na^+ (Dowex 50WX8). The yield was determined by absorption at 260 nm. The product was obtained in 3% yield (0.001 mmol). ε_{536} = 375 M^{-1} cm^{-1}. ^{31}P NMR (101 MHz, D_2O): δ −20.2 (P_β), −10.5 (P_γ), −4.6 (P_α). HRMS (ESI): $[M - H]^-$ calcd for $C_{25}H_{23}N_9O_{15}P_3^-$ m/z 782.0531, found m/z 782.0516.

Synthesis of 5-(1-Trifluoroacetamidoprop-3-yl)uridine (12).[34] In a dry, argon-flushed flask, **9**[34] (250 mg, 0.66 mmol, 1 equiv) together with $Pd(OH)_2$ (50 mg of 20 wt % on carbon, 0.07 mmol, 0.11 equiv) was dried in vacuo overnight. After suspension in abs. MeOH (5 mL), $HSiEt_3$ (1.05 mL, 6.63 mmol, 10.0 equiv) was added and the mixture stirred at room temperature for 24 h. The crude reaction mixture was filtered with Celite and concentrated under reduced pressure. The crude product was purified by column chromatography (1:1 CH_2Cl_2/acetone). The white solid product was obtained in 79% yield (200 mg, 0.52 mmol). R_f (1:1 CH_2Cl_2/acetone) = 0.35. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.31 (s, 1 H, N-H), 9.41 (t, J = 5.8 Hz, 1 H, N-H), 7.69 (s, 1 H, 6-H), 6.16 (t, J = 6.8 Hz, 1 H, 1′-H), 5.23 (d, J = 4.0 Hz, 1 H, 3′-OH), 5.02 (t, J = 5.2 Hz, 1 H, 5′-OH), 4.24 (s, 1 H, 4′-H), 3.76 (q, J = 3.4 Hz, 1 H, 3′-H), 3.57 (m, 2 H, 5′-H), 2.13 (m, 6 H, CH_2), 1.64 (m, 2 H, 2′-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 163.3 (4-C), 156.8 (q, CO-CF_3), 150.8 (2-C), 136.9 (6-C), 117.6 (CF_3), 113.1 (5-C), 87.8 (1′-C), 84.4 (4′-C), 70.9 (3′-C), 61.8 (5′-C), 46.2 (2′-C), 39.2 (CH_2), 27.6 (CH_2), 24.3 (CH_2). MS (EI): m/z 382.2 (18%) $[M + H]^+$. HRMS (EI): $[M + H]^+$ calcd for $C_{14}H_{19}F_3N_3O_6^+$ m/z 382.1220, found m/z 382.1221.

Synthesis of 5-(1-Trifluoroacetamidoprop-3-yl)uridine 13.[34] **12** (288 mg, 1.01 mmol) was dissolved in concd aq NH_3 (20 mL) and stirred for 17 h at room temperature. The solvent was removed under reduced pressure, and the remaining residue was co-evaporated with EtOH (three times). The white product was obtained in quantitative yield. The product was used without further purification. A small amount was purified by reversed phase FC (gradient from H_2O to 5:1

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527–7538

H_2O/MeCN) for analytical studies. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.73 (s, 1 H, Ar-H), 6.16 (t, J = 6.8 Hz, 1 H, H-1′), 5.27 (br, 1 H, O*H*-3′), 4.28−4.21 (m, 1H, H-3′), 3.78 (q, J = 3.5 Hz, 1H, H-4′), 3.63−3.52 (m, 2H, H-5′), 2.76 (t, J = 7.7 Hz, 2H, N-CH_2), 2.26 (t, J = 7.4 Hz, 2H, -CH_2), 2.09 (td, J = 6.1, 5.6, 3.2 Hz, 2H, H-2′), 1.71 (pd, J = 7.3, 1.7 Hz, 2H, -CH$_2$-CH_2-CH$_2$-). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.5, 150.3, 136.8, 128.3, 112.2, 87.4, 84.0, 70.4, 61.3, 38.4, 26.2, 23.5. MS (EI): m/z 286.2 (15%). HRMS (EI): [M + H]$^+$ calcd for $C_{12}H_{20}N_3O_5^+$ m/z 286.1397, found m/z 286.1399.

Synthesis of 5-[6-(6-Methyl-1,2,4,5-tetrazin-3-yl)nicotinoyl]-uridine (6). In a dry, argon-flushed flask, **13** (114 mg, 0.53 mmol, 1.0 equiv), HOBt (7.10 mg, 0.050 mmol, 0.1 equiv), and HBTU (219 mg, 0.58 mmol, 1.1 equiv) were dried in vacuo overnight. In a separate dry and argon-flushed flask, **14**[31] (150 mg, 0.53 mmol, 1.0 equiv) was dried in vacuo overnight. To the first flask were added DMF (10 mL) and (iPr)$_2$NEt (121 μL, 91.7 mg, 0.70 mmol, 1.35 equiv). The mixture was stirred at room temperature for 10 min. **13** was dissolved in DMF (4 mL), added to the reaction mixture, and stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude product purified by column chromatography (10:1 CH_2Cl_2/MeOH). The purple and solid product were obtained in 49% yield (125 mg, 0.26 mmol). R_f (8:1 CH_2Cl_2/MeOH) = 0.39. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.29 (s, 1 H, H-3), 9.26 (d, J = 2.1 Hz, 1 H, Ar-H), 8.90 (t, J = 5.7 Hz, 1 H, N-*H*), 8.60 (d, J = 8.2 Hz, 1 H, Ar-H), 8.47 (dd, J = 8.3, 2.2 Hz, 1 H, Ar-H), 7.74 (s, 1 H, H-6), 6.18 (t, J = 6.8 Hz, 1 H, H-1′), 5.24 (s, 1 H, 3′-O*H*), 5.04 (s, 1 H, 5′-O*H*), 4.26 (dt, J = 5.9, 3.1 Hz, 1 H, H-3′), 3.77 (q, J = 3.5 Hz, 1 H, H-4′), 3.64−3.55 (m, 2 H, H-5′), 3.10 (dd, J = 18.0, 7.4 Hz, 2 H, N-C$_2$), 3.06 (s, 3 H, CH_3), 2.28 (p, J = 7.5 Hz, 2H, -CH_2), 1.74 (p, J = 6.9 Hz, 2 H, CH$_2$-CH_2-CH$_2$). ^{13}C NMR (126 MHz, DMSO-d_6): δ 167.7, 164.1, 163.4, 162.9, 152.0, 150.3, 149.2, 136.5, 136.4, 131.9, 123.3, 112.9, 87.3, 83.9, 70.4, 61.3, 38.7, 27.8, 24.0, 21.0. HRMS (EI): [M + H]$^+$ calcd for $C_{21}H_{25}N_8O_6^+$ m/z 485.1892, found m/z 485.1892.

Synthesis of 5-[6-(6-Methyl-1,2,4,5-tetrazin-3-yl)nicotinoyl]-uridine 5′-Triphosphate (2). In a dry, argon-flushed flask, **6** (50 mg, 0.10 mmol, 1.0 equiv) and proton sponge (34 mg, 0.16 mmol, 1.5 equiv) were dried in vacuo overnight. Trimethylphosphate (0.95 mL) was added. The mixture was cooled to −5 °C. $POCl_3$ (10 μL, 17 mg, 0.11 mmol, 1.05 equiv) was added rapidly. After the mixture had been stirred at −5 °C for 5 h, $POCl_3$ (0.4 equiv) was added. After the mixture had been stirred for 1 h, nBu$_3$N (92 μL, 72 mg, 0.39 mmol, 4.0 equiv) and (nBu$_3$NH)$_2$P$_2$O$_7$ (1.24 mL of a 0.4 M solution in DMF, 0.62 mmol, 6.2 equiv) were added and the ice bath was removed. After 15 min, the reaction mixture was slowly (over 40 min) dropped into TEAB buffer (6.0 mL, 0.1 M, pH 7.5). After the mixture had been stirred at room temperature for 3.5 h, the solution was frozen and lyophilized. The crude product was purified by reverse phase HPLC using TEAB (50 mM, pH 6.8) as the eluent. The product-containing fractions were freeze-dried. The yield was determined by absorption at 260 nm. The product was obtained in 16% yield (0.017 mmol). ε_{260} = 14700 M^{-1} cm^{-1}. ^{31}P NMR (162 MHz, D_2O): δ −7.85, −11.11, −21.39. MS (MALDI): m/z 722.8 [M − H]$^-$.

Synthesis of 5-(4-{2-[4-*N*,*N*-(Dimethylamino)phenyl]-2*H*-tetrazol-5-yl}benzoyl)uridine (7). In a dry, argon-flushed flask, **15**[33] (108 mg, 0.35 mmol, 1.4 equiv), HOBt (4.74 mg, 0.040 mmol, 0.1 equiv), and HBTU (146 mg, 0.39 mmol, 1.1 equiv) were dried in vacuo overnight. In a separate dry and argon-flushed flask, **13** (100 mg, 0.35 mmol, 1.0 equiv) was dried in vacuo overnight. Ten milliliters of DMF was added to the first flask, followed by 0.12 mL of (iPr)$_2$NEt (90.7 mg, 0.70 mmol, 2.0 equiv). The mixture was stirred at room temperature for 20 min. **13** was dissolved in DMF (5 mL) and added, and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (20:1 CH_2Cl_2/MeOH). The slightly yellow and solid product was obtained in 28% yield (56.2 mg). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.3 (s, 1 H, H-3), 8.65 (t, J = 5.7 Hz, 1 H, N-*H*), 8.22 (d, J = 8.4 Hz, 2 H, Ar-H), 8.04 (d, J = 8.4 Hz, 2 H, Ar-H), 7.95 (d, J = 9.1 Hz, 2 H, Ar-H), 7.74 (s, 1 H, H-6), 6.92 (d, J = 9.2 Hz, 2 H, Ar-H), 6.18 (t, J = 6.8 Hz, 1 H, H-1′), 5.23 (d, J = 4.3 Hz, 1 H, H-3′), 5.04 (t, J = 5.2 Hz, 1 H, 5′-O*H*), 4.25 (m, 1 H, 3′-O*H*), 3.77 (q, J = 3.6 Hz, 1 H, H-4′), 3.65−3.52 (m, 2 H, H-5′), 3.17−2.92 (m, 8 H, N-CH_2), 2.33−2.19 (m, 2 H, H-2′), 2.19−2.01 (m, 2 H, CH_2), 1.77−1.62 (m, 2 H, CH_2). ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.9, 163.9, 151.7, 150.8, 136.9, 129.5, 128.6, 128.3, 127.6, 126.8, 125.9, 124.9, 121.6, 119.6, 113.5, 112.5, 110.2, 87.8, 84.4, 70.9, 61.8, 54.0, 46.1, 42.2, 28.4, 24.5, 19.3, 18.6. MS (FAB): m/z 577.1 (25%). HRMS (EI): [M + H]$^+$ calcd for $C_{28}H_{33}N_8O_6^+$ m/z 577.2518, found m/z 577.2518.

Synthesis of 5-(4-{2-[4-*N*,*N*-(Dimethylamino)phenyl]-2*H*-tetrazol-5-yl}benzoyl)uridine 5′-Triphosphate (4). In a dry, argon-flushed flask, **7** (50 mg, 0.09 mmol, 1.0 equiv) and proton sponge (28 mg, 0.13 mmol, 1.5 equiv) were dried in vacuo overnight. Trimethylphosphate (0.95 mL) was added, and the mixture was cooled to −5 °C, before $POCl_3$ (8.47 μL, 14.0 mg, 0.09 mmol, 1.05 equiv) was added rapidly. After the mixture had been stirred at −5 °C for 5 h, $POCl_3$ (0.4 equiv) was added. After the mixture had been stirred for 1 h, nBu$_3$N (90.7 μL, 70.8 mg, 0.38 mmol, 4.4 equiv) and (nBu$_3$NH)$_2$P$_2$O$_7$ (1.04 mL of a 0.4 M solution in DMF, 0.62 mmol, 6.0 equiv) were added and the ice bath was removed. After 15 min, the reaction mixture was slowly (over 40 min) dropped into TEAB buffer (6.0 mL, 0.1 M, pH 7.0). After the mixture had been stirred at room temperature for 3.5 h, the solution was lyophilized. The crude product was purified by reverse phase HPLC using TEAB buffer (50 mM, pH 6.8) and MeCN as eluents. The product-containing fractions were freeze-dried. The yield was determined by absorption at 260 nm. The product was obtained in 17% yield (0.015 mmol). ε_{260} = 10800 M^{-1} cm^{-1}. ^{31}P (202 MHz, D_2O): δ −10.58 (d, 1 P, J = 20.8 Hz), −11.66 (d, 1 P, J = 20.6 Hz), −23.28 (t, 1 P, J = 20.0 Hz). MS (MALDI-TOF): m/z 785.0 [M − N$_2$]$^+$.

Synthesis of 2,2,2-Trifluoro-*N*-(prop-2-yn-1-yl)acetamide (18). In a dried, argon-flushed flask, **17**[38] (200 mg, 1.28 mmol, 1.0 equiv) was added to a solution of CDI (0.250 g, 1.54 mmol, 1.2 equiv) in abs. THF (4 mL). This mixture was stirred at room temperature for 3 h. (iPr)$_2$NEt (2.23 mL, 1.65 g, 12.8 mmol, 10.0 equiv) and propargylamine (0.16 mL, 0.14 g, 2.56 mmol, 2.0 equiv) were added. This mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (10:1 hexane/EtOAc). The product was obtained as a yellowish oil in 98% yield (0.30 g, 1.26 mmol). R_f (4:1 hexane/EtOAc) = 0.61. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.88 (br, 1 H, alkynyl-H), 3.97−3.92 (m, 2 H, propargyl-CH_2), 3.89−3.86 (m, 2 H, cyclopropene-CH_2), 2.21 (s, 1 H, N-*H*), 2.17 (s, 3 H, cyclopropene-CH_3), 1.52 (t, J = 5.6 Hz, 1 H, H-3), 0.14 (s, 9 H, CH_3-Si). ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.6 (C═O), 134.5 (C-1), 111.1 (C-2), 80.2 (alkyne), 74.03 (alkyne), 71.45 (cyclopropene-CH_2), 30.9 (C-3), 18.6 (propargyl-CH$_2$), 13.33 (cyclopropene-CH$_3$), −1.13 (CH_3-Si). MS (EI): m/z 237.2 (46%) [M]$^+$. HRMS (EI): [M]$^+$ calcd for $C_{12}H_{19}NO_2Si$ m/z 237.1185, found m/z 237.1180.

Synthesis of [2-Methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl]methyl 3-[1-Prop-2-yn-1-(uridin-5-yl)]carbamate (19). In a dried flask and under argon, **16** (552 mg, 1.56 mmol, 1.0 equiv) and PPh_3 (65.5 mg, 0.25 mmol, 0.16 equiv) were dissolved in abs. DMF (20 mL) and dry NEt_3 (20 mL). **18** (462 mg, 1.95 mmol, 1.25 equiv), CuI (29.6 mg, 0.156 μmol, 0.10 equiv), and Pd(OAc)$_2$ (28.0 mg, 0.125 mmol, 0.08 equiv) were added, and the mixture was stirred at room temperature for 24 h. After the evaporation of the solvent in vacuo, the crude product was purified by reverse phase column chromatography with water and MeCN as eluents (gradient from 0 to 50% MeCN over 50 min). After lyophilization, the expected product was obtained as a white foam with a yield of 35% (253 mg, 0.55 mmol). R_f (10:1 CH_2Cl_2/MeOH) = 0.45. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.35 (br, 1 H, H-3), 8.13 (s, 1 H, H-6), 7.54 (t, J = 5.5 Hz, 1 H, carbamate-N*H*), 6.10 (t, J = 6.7 Hz, 1 H, H-1′), 5.25 (d, J = 3.9 Hz, 1 H, 5′-O*H*), 5.09 (t, J = 4.4 Hz, 1 H, 3′-O*H*), 4.22 (p, J = 3.9 Hz, 1 H, H-4′), 3.97 (d, J = 5.8 Hz, 2 H, alkynyl-CH_2), 3.85−3.78 (m, 1 H, cyclopropene-CH_2), 3.72 (dd, J = 11.1, 5.5 Hz, 1 H, H-3′), 3.62−3.52 (m, 2 H, H-2′), 2.17 (s, 3 H, cyclopropene-CH_3), 2.11 (dd, J = 6.7, 4.5 Hz, 2 H, H-5′), 1.45 (t, J = 5.4 Hz, 1 H, cyclopropene-H), 0.14 (s, 9 H, CH_3-Si). ^{13}C NMR (126 MHz, DMSO-d_6): δ 161.6 (uracil-C), 156.3 (uracil-C), 156.3 (carbamate-C═O), 149.5 (C-6), 143.6 (cyclo-

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

propene-C-1), 134.6 (cyclopropene-C-2), 110.3 (uracil), 98.2 (C-1′), 90.1 (alkyne-C), 87.7 (C-4′), 84.7 (C-3′), 72.2 (alkyne-C), 70.3 (C-5′), 61.1 (cyclopropene-CH_2), 40.1 (C-2′), 30.6 (alkynyl-CH_2), 18.4 (cyclopropene-C-3), 13.1 (cyclopropene-CH_3), −1.2 (CH_3-Si). MS (ESI): m/z 462.18 (17%) $[M - H]^-$, 498.16 (100%) $[M + 2H_2O]^-$. HRMS (ESI): $[M - H]^-$ calcd for $C_{21}H_{28}N_3O_7Si^-$ m/z 462.1702, found m/z 462.1713.

Synthesis of (2-Methylcycloprop-2-en-1-yl)methyl 3-[1-Prop-2-yn-1-(uridin-5-yl)]carbamate (8). In a dried, argon-flushed flask, **19** (70.0 mg, 0.151 mmol, 1.0 equiv) was dissolved in abs. DMF (0.5 mL). In a similarly dried flask, TBAF hydrate (39.5 mg, 0.151 mmol, 1.0 equiv) was dissolved under an argon atmosphere in dry THF (0.5 mL) and added to the reaction mixture. This was stirred at room temperature for 14 h. After evaporation of the solvent under reduced pressure, the crude product was purified by column chromatography (20:1 CH_2Cl_2/MeOH, 0.5% NEt_3). The product was dried in vacuo and was obtained as a white foam in 99% yield (58.5 mg, 0.149 mmol). R_f (10:1 CH_2Cl_2/MeOH) = 0.24. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.51 (br, 1 H, H-3), 8.14 (s, 1 H, H-6), 7.61−7.49 (m, 1 H, cyclopropene-H-3), 6.87 (s, 1 H, carbamate-N*H*), 6.10 (t, J = 6.7 Hz, 1 H, H-1′), 5.23 (s, 1 H, 5′-O*H*), 5.08 (s, 1 H, 3′-O*H*), 4.25−4.19 (m, 1 H, H-4′), 4.00−3.96 (m, 2 H, alkynyl-CH_2), 3.83−3.76 (m, 2 H, H-2′), 3.58 (q, J = 11.1 Hz, 2 H, H-3′), 3.38−3.25 (m, 2 H, cyclopropene-CH_2), 2.14−2.08 (m, 5 H, cyclopropene-CH_3, H-5′), 1.23 (t, J = 7.3 Hz, 1 H, cyclopropene-H-3). ^{13}C NMR (126 MHz, DMSO-d_6): δ 162.3 (uracil-C), 157.0 (uracil-C), 156.9 (carbamate-C), 150.1 (C-6), 144.3 (cyclopropene-C-1), 102.9 (uracil-C), 98.9 (C-1′), 90.7 (alkyne-C), 88.3 (C-4′), 85.4 (C-3′), 74.9 (cyclopropene-C-2), 72.0 (alkyne-C), 71.0 (C-5′), 61.7 (cyclopropene-CH_2), 46.4 (C-2′), 31.3 (alkynyl-CH_2), 17.4 (cyclopropene-C-3), 13.4 (cyclopropene-CH_3). MS (ESI): m/z 390.13 (53%) $[M - H]^-$, 781.27 (100%) $[2M - H]^-$. HRMS (ESI): $[M - H]^-$ calcd for $C_{18}H_{20}N_3O_7^-$ m/z 390.1307, found m/z 390.1307.

Synthesis of [2-Methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl]methyl 3-[1-Prop-2-yn-1-(uridin-5′-triphosphate-5-yl)]-carbamate (3). In a dried, argon-flushed flask, **8** (15.0 mg, 38.3 μmol, 1.0 equiv) and freshly mortared proton sponge (12.2 mg, 57.5 μmol, 1.5 equiv) were dried overnight. Trimethylphosphate and nBu$_3$N were dried overnight over molecular sieves (under argon). **8** and proton sponge were dissolved in trimethylphosphate (0.308 mL) and cooled to −15 °C using a salt/ice bath. $POCl_3$ (3.76 μL, 6.18 mg, 40.3 μmol, 1.05 equiv) was quickly added, and this mixture was cooled and stirred for 4 h. Additional $POCl_3$ (1.43 μL, 0.4 equiv) was added, and the solution was stirred at −15 °C for an additional 1 h. A solution of dried (nBu$_3$NH)$_2$P$_2$O$_7$ (126 mg, 230 μmol, 6.0 equiv) and nBu$_3$N (29.8 μL) in abs. DMF (402 μL) was added to the mixture, and the cooling bath was removed. This solution was stirred for 15 min and then added (dropwise) over a period of 40 min to TEAB buffer (3 mL, 0.1 M, pH 7.5). This solution was stirred at room temperature for 4 h and lyophilized. The crude product was purified by reverse phase HPLC using TEAB buffer (0.05 M, pH 7.8) and MeCN as eluents (gradient from 0 to 50% MeCN over 50 min). The yield was determined by absorption at 260 nm. The product was obtained as white foam in 9% yield (3.45 μmol). ε_{260} = 3300 M^{-1} cm^{-1}. ^{31}P (202 MHz, D_2O): δ −7.75 to −8.42 (m, 1 P), −11.37 (d, 1 P, J = 20.1 Hz), −22.8 (t, 1 P, J = 20.2 Hz). MS (MALDI-TOF): m/z 627.0 $[M - H]^-$.

Primer Extension Experiments. The reaction mixture (9.5 μL) containing primer (1.5 μM), template (1.8 μM), and buffer was incubated at 90 °C for 10 min. After the mixture had cooled to room temperature over 1 h, the modified nucleoside triphosphate, other dNTPs (400 μM each), and polymerase (0.5 μL; see PAGE analysis for concentration) were added to a final reaction volume of 20 μL. Primer extension was conducted at cited temperatures and cited reaction times as mentioned in the figure captions of PAGE analysis. After the mixture had cooled to room temperature, EDTA-containing loading buffer (20 μL of 20 mM, pH 8.0, 89 mM TRIS, 89 mM boric acid, 12% Ficoll, 0.01% bromophenol, 0.02% xylene cyanol FF, 7.0 M urea) was added to stop further extension. For postsynthetic labeling, the primer extension experiments were conducted as described above without adding loading buffer at the end. To stop further elongation, the reaction mixture was desalted by illustra MicroSpin G-25 Columns from GE Healthcare Life Sciences and then freeze-dried.

Postsynthetic Bioorthogonal Labeling. In the case of iEDDA reactions with tetrazine-modified DNA, the oligonucleotides were dissolved in highly purified water (10 μM, with respect to the fully elongated primer strand). The modified rhodamine dyes were dissolved in DMSO (10 mM for **20** and 5 mM for **21**). The labeling reactions were performed by mixing solutions of oligonucleotides (2 μL) and dye (2 μL) and shaking them at room temperature for the times mentioned. The reaction was stopped by adding loading buffer [4 μL (see above)]. The whole volume was immediately loaded on a denaturing polyacrylamide gel. The freeze-dried residue was dissolved in highly purified water to produce a 750 nM solution (with respect to the fully elongated primer strand). This solution was spread into fractions (20 μL) and mixed with the corresponding tetrazine-modified dye **22** (1 μL of a 12.5 mM solution in DMSO). The labeling was performed at different reaction times at room temperature and was stopped by adding loading buffer [20 μL (see above)] and loading on the denaturing polyacrylamide gel. PAGE was performed as described in the following section. The "photoclick" reaction was performed by adding 40 μL of a 252 μM solution of the sulfonyl-modified Cy3 dye to 300 μL of a crude primer extension mixture (concentrations described above) followed by irradiation at 365 nm for the specified times with two high-powered LEDs at 20 °C. After irradiation, a 20 μL sample was removed and 20 μL of loading buffer was added. The experiments were analyzed by PAGE.

Denaturing Polyacrylamide Gel Electrophoresis. The analysis of primer extension and bioorthogonal labeling experiments was performed by denaturing polyacrylamide gel electrophoresis (PAGE). The freshly prepared gel mixtures [12% acrylamide/bisacrylamide (19:1), urea (8.3 M), TEMED (2.65 mM), EDTA (2 mM, pH 8.0), 89 mM TRIS base, and 89 mM boric acid] were polymerized by adding ammonium persulfate (4 mM). PAGE analysis was performed in a Sequi-Gen GT Sequencing Cell (38 cm × 50 cm) with a PowerPac HV from Bio-Rad at 50 °C and 45 W for ∼60 min. As an electrolyte, a TBE buffer [89 mM TRIS base, 89 mM boric acid, and 2 mM EDTA (pH 8.0)] was used. For the visualization of the synthesized oligonucleotides, a Stella 8300 fluorescence imager from Raytest was used. The fluoresceine and rhodamine dyes were irradiated from above by the use of different LEDs (λ_{exc} = 470 ± 20, 540 ± 10, or 630 ± 10 nm). The emission was detected by a CCD camera (cooled to −20 °C) with different emission filters (535 ± 20, 605 ± 10, and 700 ± 17.5 nm). The acquired pictures were analyzed with an AIDA Image Analyzer software from Raytest. For quantification, the 1D Multi Labeling mode was used.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01205.

Standing start experiments with **2** and **3**, images of ^{1}H NMR spectra, ^{13}C NMR spectra, and mass analysis of compounds **5**, **1**, **12**, **13**, **6**, **2**, **7**, **4**, **18**, **19**, **8**, and **3** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Wagenknecht@kit.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from DFG (Project ERACHEM Wa 1386/15-1 and GRK 2039-1) and by KIT is gratefully acknowlegded. D.P. thanks the Carl-Zeiss-Stiftung for a Ph.D. fellowship. This

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538

work was supported by the Hungarian Scientific Research Fund (OTKA, NN-110214) and the "Lendület" Program of the Hungarian Academy of Sciences (LP2013-55/2013).

REFERENCES

(1) Späte, A.-K.; Bußkamp, H.; Niederwieser, A.; Schart, V. F.; Marx, A.; Wittmann, V. *Bioconjugate Chem.* **2014**, *25*, 147.
(2) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873.
(3) Chang, P. V.; Bertozzi, C. R. *Chem. Commun.* **2012**, *48*, 8864.
(4) Dean, K. M.; Palmer, A. E. *Nat. Chem. Biol.* **2014**, *10*, 512.
(5) Merkel, M.; Peewasan, K.; Arndt, S.; Ploschik, D.; Wagenknecht, H.-A. *ChemBioChem* **2015**, *16*, 1541.
(6) Gramlich, P. M.; Wirges, C. T.; Manetto, A.; Carell, T. *Angew. Chem., Int. Ed.* **2008**, *47*, 8350.
(7) Amblard, F.; Cho, J. H.; Schinazi, R. F. *Chem. Rev.* **2009**, *109*, 4207.
(8) El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388.
(9) Seela, F.; Ingale, S. A. *J. Org. Chem.* **2010**, *75*, 284.
(10) Fauster, K.; Hartl, M.; Santner, T.; Aigner, M.; Kreutz, C.; Bister, K.; Ennifar, E.; Micura, R. *ACS Chem. Biol.* **2012**, *7*, 581.
(11) Aigner, M.; Hartl, M.; Fauster, K.; Steger, J.; Bister, K.; Micura, R. *ChemBioChem* **2011**, *12*, 47.
(12) Beyer, C.; Wagenknecht, H.-A. *Chem. Commun.* **2010**, *46*, 2230.
(13) Weisbrod, S. H.; Marx, A. *Chem. Commun.* **2007**, 1828.
(14) Hong, V.; Steinmetz, N. F.; Manchester, M.; Finn, M. G. *Bioconjugate Chem.* **2010**, *21*, 1912.
(15) Yang, M.; Li, J.; Chen, P. R. *Chem. Soc. Rev.* **2014**, *43*, 6511.
(16) Kennedy, D. C.; McKay, C. S.; Legault, M. C. B.; Danielson, D. C.; Blake, J. A.; Pegoraro, A. F.; Stolow, A.; Mester, Z.; Pezacki, J. P. *J. Am. Chem. Soc.* **2011**, *133*, 17993.
(17) Ren, X.; El-Sagheer, A.; Brown, T. *Analyst* **2015**, *140*, 2671.
(18) Baccaro, A.; Weisbrod, S. H.; Marx, A. *Synthesis* **2007**, *2007*, 1949.
(19) Ren, X.; Gerowska, M.; El-Sagheer, A. H.; Brown, T. *Bioorg. Med. Chem.* **2014**, *22*, 4384.
(20) Borsenberger, V.; Howorka, S. *Nucleic Acids Res.* **2009**, *37*, 1477.
(21) Asare-Okai, P. N.; Agustin, E.; Fabris, D.; Royzen, M. *Chem. Commun.* **2014**, *50*, 7844.
(22) Bußkamp, H.; Batroff, E.; Niederwieser, A.; Abdel-Rahman, O. S.; Winter, R. F.; Wittmann, V.; Marx, A. *Chem. Commun.* **2014**, *50*, 10827.
(23) Hottin, A.; Marx, A. *Acc. Chem. Res.* **2016**, *49*, 418.
(24) Debets, M. F.; van Hest, J. C. M.; Rutjes, F. P. J. T. *Org. Biomol. Chem.* **2013**, *11*, 6439.
(25) Lang, K.; Chin, J. W. *ACS Chem. Biol.* **2014**, *9*, 16.
(26) Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. *ACS Chem. Biol.* **2014**, *9*, 592.
(27) Ramil, C. P.; Lin, Q. *Chem. Commun.* **2013**, *49*, 11007.
(28) Csérep, G. B.; Herner, A.; Kele, P. *Methods Appl. Fluoresc.* **2015**, *3*, 042001.
(29) Ramil, C. P.; Lin, Q. *Curr. Opin. Chem. Biol.* **2014**, *21*, 89.
(30) Karver, M. R.; Weissleder, R.; Hilderbrand, S. A. *Bioconjugate Chem.* **2011**, *22*, 2263.
(31) Cserép, G. B.; Demeter, O.; Bätzner, E.; Kállay, M.; Wagenknecht, H.-A.; Kele, P. *Synthesis* **2015**, *47*, 2738.
(32) Eördögh, Á.; Steinmeyer, J.; Peewasan, K.; Schepers, U.; Wagenknecht, H.-A.; Kéle, P. *Bioconjugate Chem.* **2016**, *27*, 457.
(33) Arndt, S.; Wagenknecht, H.-A. *Angew. Chem., Int. Ed.* **2014**, *53*, 14580.
(34) Nuzzolo, M.; Grabulosa, A.; Slawin, A. M. Z.; Meeuwenoord, N. J.; van der Marel, G. A.; Kamer, P. C. J. *Eur. J. Org. Chem.* **2010**, *2010*, 3229.
(35) Schoch, J.; Wiessler, M.; Jäschke, A. *J. Am. Chem. Soc.* **2010**, *132*, 8846.
(36) Braun, K.; Wiessler, M.; Ehemann, V.; Pipkorn, R.; Spring, H.; Debus, J.; Didinger, B.; Koch, M.; Muller, G.; Waldeck, W. *Drug Des., Dev. Ther.* **2008**, *5576*, 289.
(37) Asare-Okai, P. N.; Agustin, E.; Fabris, D.; Royzen, M. *Chem. Commun.* **2014**, *50*, 7844.
(38) Patterson, D. M.; Nazarova, L. A.; Xie, B.; Kamber, D. N.; Prescher, J. A. *J. Am. Chem. Soc.* **2012**, *134*, 18638.
(39) Ludwig, J. *Acta Biochim. Biophys. Acad. Sci. Hung.* **1981**, *16*, 131.
(40) Wenge, U.; Ehrenschwender, T.; Wagenknecht, H.-A. *Bioconjugate Chem.* **2013**, *24*, 301.
(41) Kong, H.; Kucera, R. B.; Jack, W. E. *J. Biol. Chem.* **1993**, *268*, 1965.
(42) Perler, F. B.; Comb, D. G.; Jack, W. E.; Moran, L. S.; Qiang, B.; Kucera, R. B.; Benner, J.; Slatko, B. E.; Nwankwo, D. O.; Hempstead, S. K. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 5577.
(43) Jannasch, H. W.; W, C. O.; Molyneaux, S. J.; Langworthy, T. A. *Appl. Environ. Microbiol.* **1992**, 3472.
(44) Kermekchiev, M. B.; Kirilova, L. I.; Vail, E. E.; Barnes, W. M. *Nucleic Acids Res.* **2009**, *37*, e40.
(45) Huber, M. C.; Schreiber, A.; von Olshausen, P.; Varga, B. R.; Kretz, O.; Joch, B.; Barnert, S.; Schubert, R.; Eimer, S.; Kele, P.; Schiller, S. M. *Nat. Mater.* **2014**, *14*, 125.
(46) Kamber, D. N.; Nazarova, L. A.; Liang, Y.; Lopez, S. A.; Patterson, D. M.; Shih, H.-W.; Houk, K. N.; Prescher, J. A. *J. Am. Chem. Soc.* **2013**, *135*, 13680.
(47) Yang, J.; Šečkutė, J.; Cole, C. M.; Devaraj, N. K. *Angew. Chem., Int. Ed.* **2012**, *51*, 7476.
(48) Yang, J.; Liang, Y.; Seckute, J.; Houk, K. N.; Devaraj, N. K. *Chem. - Eur. J.* **2014**, *20*, 3365.
(49) Clovis, J. S.; Eckell, A.; Huisgen, R.; Sustmann, R. *Chem. Ber.* **1967**, *100*, 60.
(50) Wang, Y.; Hu, W. J.; Song, W.; Lim, R. K. V.; Lin, Q. *Org. Lett.* **2008**, *10*, 3725.

DOI: 10.1021/acs.joc.6b01205
J. Org. Chem. 2016, 81, 7527−7538