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1,2,4-triazine-modified 2'-deoxyuridine triphosphate for efficient bioorthogonal fluorescent labeling of DNA

Krisana Peewasan[a] and Hans-Achim Wagenknecht*[a]

Abstract: In order to establish the Diels-Alder reaction with inverse electron demand for postsynthetic DNA modification a 1,2,4-triazine-modified 2'-deoxyuridine triphosphate was synthesized. The bioorthogonally reactive 1,2,4-triazine group was attached at the 5-position of 2'-deoxyuridine by a flexible alkyl linker to facilitate its acceptance by DNA polymerases. The screening of four DNA polymerases showed successful primer extensions using a mixture of dATP, dGTP, dCTP and the modified 2'-deoxyuridine triphosphate with the KOD XL and Vent polymerases. The triazine moiety was stable under the conditions of primer extension, which was evidenced by labeling with a BCN-modified rhodamine at room temperature in yields of up to 82%. Dual (and triple) modifications were successfully achieved and obtained in quantitative yields if the modification sites were separated by three base pairs. These results establish the 1,2,4-triazene as bioorthogonally reactive moiety in DNA, thereby replacing the problematic 1,2,4,5-tetrazine for postsynthetic labeling by the Diels-Alder reaction with inverse electron demand.

In the bioorthogonal toolbox for nucleic acids,[1] 1,2,4,5-tetrazines play an important role because they undergo very rapid inverse electron-demand Diels-Alder reactions in particular with strained dienophiles such as trans-cyclooctenes or bicyclo[6.1.0]nonynes (BCN).[2] These reactions proceed with the fastest rate constants ($k \leq 10^5$ $M^{-1}s^{-1}$) of the currently available bioorthogonal reactions.[3] Unfortunately, the faster reacting 1,2,4,5-tetrazines are also chemically quite labile. Especially the highly reactive tetrazines are prone to hydrolysis[3c] and react with endogenous thiols,[4] limiting their application in living cells.[4-5] Due to their lack of stability towards the rather harsh conditions of chemical DNA synthesis along with the basic conditions for the cleavage step from the solid phase, only a few examples for 1,2,4,5-tetrazine-based labeling of nucleic acids were published. We firstly reported about 1,2,4,5-tetrazine-modified DNA for on-bead fluorescent labeling using the phosphoramidite method.[6] Moreover, we developed and synthesized 2'-deoxyuridine triphosphates as DNA building blocks with covalently attached 1,2,4,5-tetrazines for primer extension using DNA polymerases. The subsequent reaction of the 1,2,4,5-tetrazine-oligonucleotide conjugates with carboxymethylmonobenzocyclooctyne- and BCN-modified fluorophores could significantly be improved by the more stable, but still reactive nicotinic acid-derived 1,2,4,5-tetrazine and by changing the key experimental conditions, mainly the pH of 7.2 and temperature of 45-55 °C, but the yield did not exceed 40%.[7] In another approach it was demonstrated that DNA bearing a 1,2,4,5-tetrazine at the 5'-end was rapidly ligated with cyclopropene-3'-modified DNA by the advances of a template-dependent ligation.[8] An alternative strategy involves the synthetic introduction of vinyl groups[9] and cyclopropenes[10] as dienophiles into nucleic acids which can be postsynthetically labeled with 1,2,4,5-tetrazine-modified dyes.

Recently, Prescher *et al.* presented 1,2,4-triazines as new class of bioorthogonal labels that react efficiently and selectively with trans-cyclooctene, but not with norbornene and cyclopropene.[11] Moreover, 1,2,4-triazines are highly stable in aqueous media. Based on these two major advantages it looked promising to further develop this biorthogonal label for nucleic acids. We report herein the synthesis of a 2'-deoxyuridine triphosphate with a 1,2,4-triazine modification in the 5-position for primer extension and subsequent fluorescent labelling of the DNA products by a BCN-modified rhodamine.

The synthesis of the 2'-deoxyuridine triphosphate **8** starts with building the 1,2,4-triazine heterocycle. The key intermediate 3-carboxy-1,2,4-triazine (**5**) was achieved by the reaction of commercially available ethyl oxamate (**1**) and Lawesson's reagent which provided the reactive intermediate **2**, and which was subsequently treated with a 1 M solution of hydrazine in THF to obtain ethyl amino(hydrazo)acetate (**3**) in 62% yield.[12] The ethyl ester **4** of 1,2,4-triazine-3-carboxylate was obtained in 74% yield by the condensation reaction of **3** with 40% aqueous glyoxal solution. Further treatment with KOH followed by 1 N HCl gave the corresponding product **5** in 76% yield.[13] The peptide coupling of aminopropyl-2'-deoxyuridine **6**[7] with 3-carboxy-1,2,4-triazine (**5**) was performed with HBtU and HOBt in 45% yield. After subsequent phosphorylation of **7** using the methodology of Ludwig *et al.*[14] the crude product **8** was purified by RP-HPLC, freeze-dried and quantified by UV/Vis absorption spectroscopy at 260 nm, which gave a yield of 15%.

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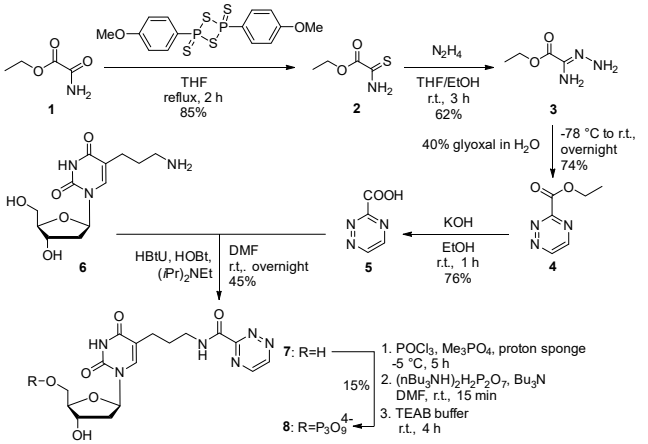

Scheme 1. Synthesis of the 1,2,4-triazine-modified 2'-deoxyuridine triphosphate **8**.

For the subsequent primer extension experiments with the 1,2,4-triazine-modified 2'-deoxyuridine triphosphate **8** the Vent(exo-), Deep Vent(exo-), Homo Klen Taq and KOD XL DNA polymerases were screened since they typically accept modifications in the 5-position.[15] The primer extension experiments were first performed using the primer **P1** that contained a fluorescein label at the 5'-terminus in order to follow the elongation product by fluorescence imaging of gels after electrophoresis using the green channel of the detector. Template **T1** was chosen for the initial screening as standing start experiments. **T2-T4** were designed for multiple incorporations of **9** in running start experiments.

Scheme 2. Templates **T1-T4** and primer **P1** for primer extension experiments.

It was necessary to perform the primer extension experiments at 64 °C in order to get the fully elongated product within 30 min reaction time (Figure 1). According to the 24 nucleotide long reference **P2** and the 35 nucleotide long reference **P3** (both with T instead of the modified dU, see Supporting Information) the gel band with the slowest mobility was assigned to the fully elongated oligonucleotide product. The small shift difference to **P3** can be assigned to the additional 1,2,4-triazine label which indicates that the modified dU moiety of **8** was successfully incorporated. It became obvious from the gel images that this primer extension worked best with the KOD XL and Vent polymerases. Additional control experiments (without natural dNTPs) were performed with the KOD XL polymerase at 64 °C (see Supporting Information). The fully elongated oligonucleotide product was only obtained if all natural dNTPs (dATP, dGTP, dCTP) and **8** were added.

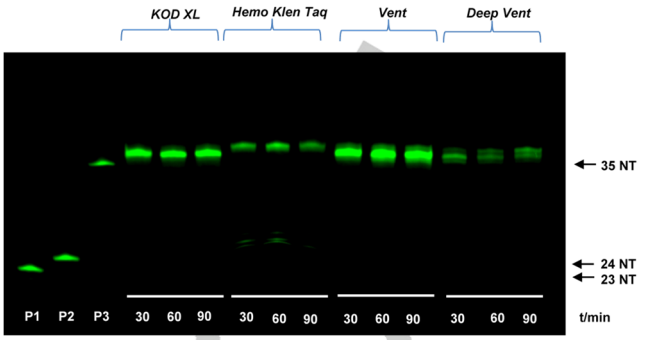

Figure 1. Standing start experiment with **P1** (750 nM)/**T1** (900 nM) and KOD XL, Hemo Klen Taq, Vent (exo-) and Deep Vent (exo-) DNA polymerase (0.25 U), in the presence of dATP, dGTP, dCTP and **8** (each 100 μM). The references **P2** (24 nucleotides) **P3** (35 nucleotides) contained both T instead of the modified dU. The fluorescence of fluorescein was detected by λ_{exc}=470±20 nm and λ_{em}=535±20 nm.

The successful incorporation of the 1,2,4-triazine-modified building block **8** into DNA was further probed by the ability of this reactive probe for postsynthetic labeling with the BCN-modified rhodamine **9** which was performed in a mixture of H_2O-DMSO (100:1) at room temperature.[7] In the initial experiments without desalting of the primer extension product, PAGE analysis indicated that the conjugated product was obtained in 74% yield after 1 h (Figure 2). The DNA-dye conjugate was imaged by the additional red channel of the detector. The yield could not further be improved by adding more dye or by extending the reaction time But the conjugation yield could be enhanced to 82% by an additional desalting step prior to the conjugation step. There is also a small amount of misincorporation detectable as second band underneath the full length product that is not reacting with the dye **9**. This assignment is additionally supported by the gel shift difference between the side product and the correct and main primer extension product that is similar to that between the primer extension product (with triazine) and the reference P3 (without triazine) in Figure 1. Degradation of the triazine function in the primer extension product is very unlikely regarding the observed high chemicals stability of the triazine during the nucleoside synthesis. Overall, this reactivity of the 1,2,4-triazine is a remarkable improvement in comparison to our recently published attempts with the 1,2,4,5- tetrazine moiety in DNA.[7]

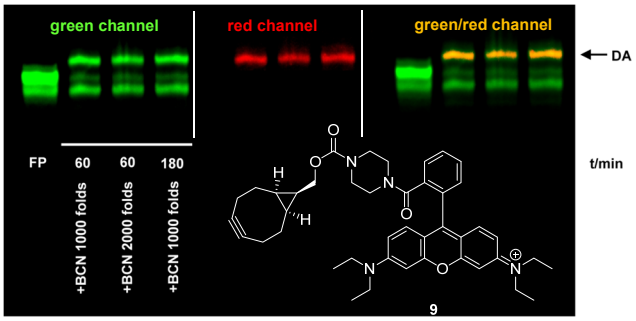

Figure 2. PAGE analysis of postsynthetic modification of the full length product (FP) with the BCN-modified dye **9** after primer extension extension with **P1** (750 nM)/**T1** (900 nM), dATP/dGTP/dCTP/**8** (each 100 μM) and KOD XL polymerase

(0.25 U). "DA" marks the Diels-Alder product; green channel (left lanes): fluorescence of fluorescein (λ_{exc}=470±20 nm, λ_{em}=535±20 nm), red channel (middle lanes): fluorescence of rhodamine (λ_{exc}=540±10 nm, λ_{em}=605±10 nm), right lanes: overlay of red and green channel.

Template **T2** was designed for double modification with the modified 2'-deoxyuridines next to each other; template **T3** for double modification with the modified dU building blocks separated from each other by 3 intervening C-G base pairs, and template **T4** for triple incorporation next to each other. First of all, the primer **P1** was successfully extended in all combinations with **T2**-**T4** using the KOD XL polymerase. It was furthermore evident from PAGE analysis (Figure 3) that the extension product with **P1**/**T2** could be labeled by **9** within 3 h and yielded an approximate 1:1 mixture of single and double labeling, probably because the modification sites were located next to each other and thereby to close to each other. This goes along with the observation that the corresponding reaction of the extension product with **P1**/**T3** and dye **9** was completed in 1 h and yielded the doubly modified bioconjugation product in nearly quantitative yield. Obviously, the steric hindrance is reduced if the modification sites are separated by three additional base pairs. Accordingly it is not surprising that PAGE analysis after the labelling reaction with the extension product of **P1**/**T3** revealed a mixture of singly, doubly and triply modified oligonucleotide in 65% total yield.

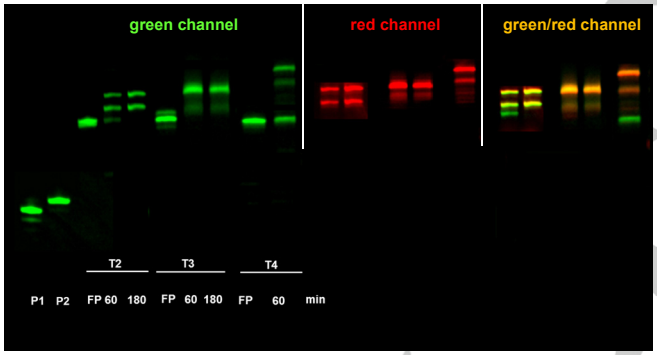

Figure 3. PAGE analysis of postsynthetic modifications of the full length product (FP) with the BCN-modified dye **9** after the primer extension with **P1**/**T2**, **P1**/**T3** and **P1**/**T4**, respectively, (primer 750 nM, template 900 nM), dATP/dGTP/dCTP/**8** (each 100 μM) and KOD XL polymerase); green channel (left): fluorescence of fluorescein (λ_{exc}=470±20 nm, λ_{em}=535±20 nm), red channel (middle): fluorescence of rhodamine (λ_{exc}=540±10 nm, λ_{em}=605±10 nm), right: overlay of red and green channel.

In conclusion, the 1,2,4-triazine-modified 2'-deoxyuridine triphosphate building block **8** was prepared in good overall yield and successfully incorporated into DNA by enzymatic primer extension. The screening of DNA polymerases showed acceptance of this modified nucleotide triphosphate especially by the KOD XL and Vent polymerases. The 1,2,4-triazine moiety was stable under the conditions of primer extension which was evidenced by labeling with the BCN-modified rhodamine **9** in yields of up to 82%. Dual modifications were achieved in quantitative yields if the modification sites were separated by three base pairs. Thereby, the 1,2,4-triazine replaces the problematic 1,2,4,5-tetrazine as bioorthogonally reactive moiety in DNA. Postsynthetic modifications of DNA (and potentially also RNA) by this type of inverse-electron demand Diels-Alder reactions as important and fast copper-free bioorthogonal reactions can now be considered as an important method of choice to introduce fluorescent probes into oligonucleotides. The important advantage of this approach is that it is only necessary to synthesize the 2'-deoxyuridine triphosphate **9** with the 1,2,4-triazine as reactive functional group that can be applied to a variety of different fluorophores. Chemoselectivity and bioorthogonality is provided by the Diels-Alder-type cycloaddition chemistry and should –in principle- allow performing this type chemistry also in living cells (as recently shown for the strain-promoted cycloaddition[16]).

Experimental Section

All experimental details are described in the Supporting Information.

Acknowledgements

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Keywords: oligonucleotide • rhodamine • primer extension • DNA polymerase • Diels-Alder reaction

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Click well: 1,2,4-Triazenes are able to replace the problematic 1,2,4,5-tetrazines for postsynthetic fluorescent labelling of DNA by Diels-Alder reactions with inverse electron demand.