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"Photoclick" Postsynthetic Modification of DNA**

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Abstract: *A new DNA building block bearing a push–pull-substituted diaryltetrazole linked to the 5-position of 2′-deoxyuridine through an aminopropynyl group was synthesized. The accordingly modified oligonucleotide allows postsynthetic labeling with a maleimide-modified sulfo-Cy3 dye, N-methylmaleimide, and methylmethacrylate as dipolarophiles by irradiation at 365 nm (LED). The determined rate constant of* $(23 \pm 7)\,M^{-1}s^{-1}$ *is remarkably high with respect to other copper-free bioorthogonal reactions and comparable with the copper-catalyzed cycloaddition between azides and acetylenes.*

Postsynthetic modification of nucleic acids is a well-established method and mainly achieved by copper-catalyzed cycloadditions between acetylenes and azides.[1,2] Although successful labeling in live cells by copper-catalyzed "click"-type reactions has been demonstrated,[3] the reliance on copper catalysis is problematic. Even trace amounts of copper ion impurities from the postsynthetic or in vivo modification represent a major drawback in terms of cytotoxicity.[4] Hence, current research from our[5] and other[6–11] groups focuses on the development of bioorthogonal and copper-free "click"-type alternatives for the modification of nucleic acids. This includes strain-promoted[5] and other 1,3-dipolar cycloadditions,[6] Diels–Alder reactions with normal[7] and inverse electron demand,[8] reductive aminations,[9] thiol-ene additions,[10] and Suzuki–Miyaura-type coupling reactions.[11] There are two metal-free bioorthogonal reactions that are good alternatives, since their reaction rates are comparable to the rates of copper-catalyzed azide–acetylene cycloadditions ($k \approx 10$–$200\,M^{-1}s^{-1}$).[12,13] The first alternative is the inverse electron demand Diels–Alder reaction of tetrazines and strained alkenes ($k \approx 1$–$10^4\,M^{-1}s^{-1}$)[8,12,14] and, the second alternative the light-induced cycloaddition between tetrazoles and activated alkenes ("photoclick" reaction, $k \approx 60\,M^{-1}s^{-1}$).[12,13]

The latter type is especially attractive since it combines the speed and specificity of a "click"-type reaction with the advantages of a photochemical process, in particular spatial and temporal control. In the 1960s, Huisgen and co-workers reported not only the photoinduced cycloaddition between 2,5-diphenyltetrazole and methyl crotonate but also the detection of the 1,3-dipolar intermediate diphenylnitrilimine.[15] Recently, Lin and co-workers further developed this type of reaction for use in water,[16] for peptide cross-linking,[17] and finally as an important method for the photoinduced modification of proteins.[18] The "photoclick" cycloaddition, however, was never used for the modification of nucleic acids, probably because of the fact that typical 2,5-diphenyltetrazoles have to be irradiated at a wavelength between 250 nm and 310 nm, which strongly overlaps with the absorption of nucleic acids. Lin and co-workers, however, described the photoactivatable diaryltetrazole **1**, which has an electron-donating dimethylamino group at one end and an electron-withdrawing carboxy group at the other end.[19] The push–pull system of this diaryltetrazole shifts the excitation to 365 nm, which is a wavelength that allows not only selective excitation outside the absorption range of the nucleic acids but also the application of LEDs as cheap and efficient light sources. Herein, we describe the new DNA building block **3**, which bears the diaryltetrazole **1** linked through an aminopropynyl group to the 5-position of 2′-deoxyuridine, together with its incorporation into an oligonucleotide by using automated phosphoramidite chemistry and subsequent "photoclick" modification with a sulfonated Cy3 dye.

The synthesis (Scheme 1) started with 5-(3″-aminopropynyl)-2′-deoxyuridine (**2**) as a precursor, which was obtained from 2′-deoxyuridine according to literature procedures.[15] The second precursor, the push–pull-substituted diphenyltetrazole **1**, was synthesized from the sulfonated hydrazone of formyl benzoic acid methyl ester and the diazonium salt of *p*-*N*,*N*-dimethylaminoaniline (see the Supporting Information). The carboxylic acid moiety of tetrazole **1** was attached to the aminopropynyl group of nucleoside **2** with the peptide-coupling reagent HBTU. The resulting nucleoside–tetrazole conjugate **3** was obtained in 83% yield and then converted into DNA building block **5** by standard procedures. **DNA1** was synthesized from this DNA building block by automated solid-phase chemistry (with an extended coupling time of 2 × 300 s for **5**) and purified by semipreparative HPLC.

Subsequently, the functional and reactive tetrazole group of **DNA1** was used in a "photoclick" postsynthetic modification with commercially available sulfo-Cy3 dye **6**, which bears a maleimide functionality at the end a short alkyl linker. Unexpectedly, the cycloaddition did neither work with nucleoside **4** in water/MeCN nor with single-stranded (ss) **DNA1** in DMSO or water/DMSO mixtures. The reaction occurred only in standard aqueous buffer solution (50 mM sodium phosphate buffer, 250 mM NaCl, pH 7), which is clearly an advantage for future in vivo applications. The reaction can be followed by UV/Vis absorption spectroscopy (Figure 1), since the characteristic absorption of the tetrazole

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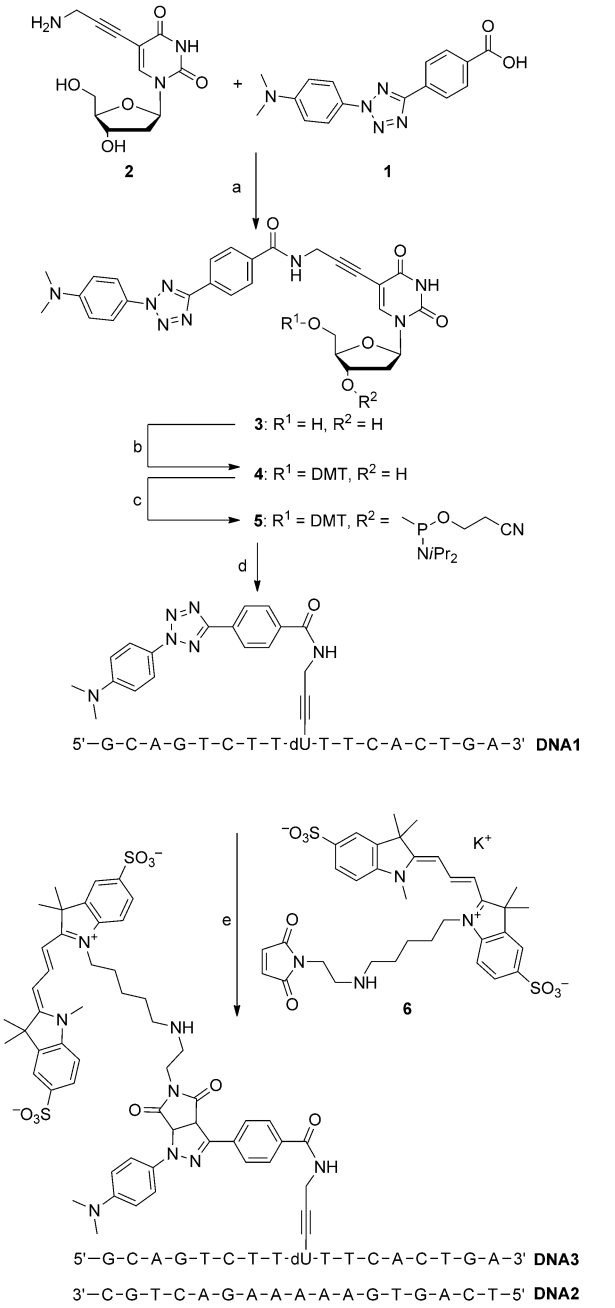

Scheme 1. Synthesis of tetrazole-modified DNA building block **5** and postsynthetic modification of **DNA1** with sulfo-Cy3 dye **6**: a) HBTU, HOBt·H_2O, $(iPr)_2NEt$, DMF, RT, 20 h; 83%; b) DMT-Cl, pyridine, RT, 3 days; 60%; c) β-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, RT, 24 h; 99%; d) solid-phase synthesis under standard conditions; e) **6**, LED $\lambda = 365$ nm, 10 mM sodium phosphate buffer, 250 mM NaCl, pH 7, 20°C, 30 min. DIPEA = diisopropylethylamine, DMT = dimethoxytrityl, HBTU = *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole.

chromophore of **DNA1** occurs as a side band between 300 nm and 400 nm, with a broad maximum at 355 nm. Irradiation of the solution by LEDs at a wavelength of 365 nm resulted in the absorption at 355 nm decreasing and the appearance of a new absorption at 400 nm, which can be assigned to the

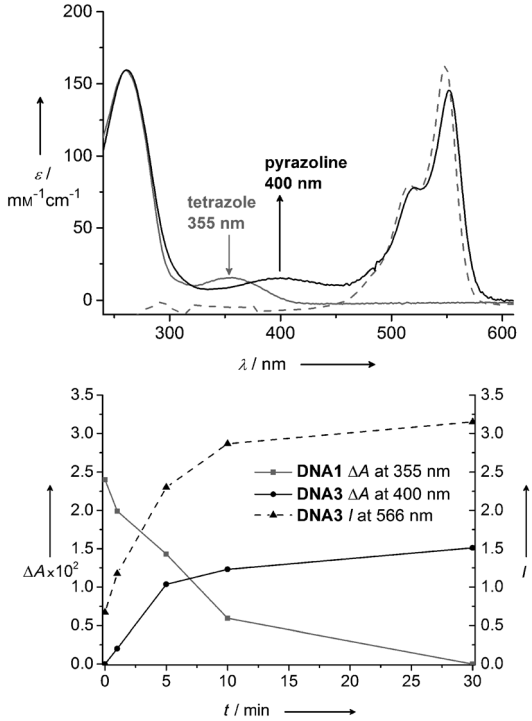

Figure 1. Top: UV/Vis absorption of starting material **DNA1** with **6** compared with that of the "photoclick" product **DNA3**; **DNA1** (2,5 μM), **6** (1.05 equiv, 2.63 μM). Bottom: Time-dependent absorption changes at 355 nm (**DNA1**) and 400 nm (**DNA3**), and the time-dependent fluorescence of **DNA3**, $\lambda_{exc} = 357$ nm. All measurements were performed in 50 mM sodium phosphate buffer, 250 mM NaCl, pH 7, 20°C.

formation of the pyrazoline chromophore in the product **DNA3**. The product was additionally identified by MALDI-TOF mass spectrometry. The isosbestic point at $\lambda_{abs} = 378$ nm (see the Supporting Information) shows that this "photoclick" modification proceeds without observable intermediates, and supports the idea that the nitrilimine intermediate is very short-lived and reacts very fast with the maleimide functionality of **6**. The modified **DNA3** was again purified by semipreparative HPLC, identified by MALDI-TOF MS, and quantified by UV/Vis absorption at $\lambda_{abs} = 260$ nm (using $\varepsilon(\mathbf{6})260\,nm = 3400\,M^{-1}cm^{-1}$) to obtain yields for the "photoclick" modification. Product formation was determined after irradiation for 30 min at 365 nm by an LED. Interestingly, the yield of purified **DNA3** increased from 8% to 15% (with 1.05 equiv **6**) if double-stranded **DNA1** was used as the starting material, and further to 34% (with 5 equiv **6**). Longer irradiation times were not productive, since photochemical degradation of the diaryltetrazole group competes with the desired photoinduced cycloaddition. The observation that double-stranded **DNA1–2** works better than single-stranded **DNA1** can be rationalized by the assumption that the regular double-helical structure makes the tetrazole functional group more accessible for the reaction with **6** than does the corresponding single strand, where the diaryltetrazole functionality is "hidden" in the random coil. The "photoclick" cycloaddition of tetrazoles to form pyrazolines is known to be a fluorogenic reaction.[20,21] However, the pyrazoline fluorescence of **DNA3** is rather weak (see the Supporting Informa-

tion). The fluorescence (upon excitation at 357 nm) is dominated by the strong fluorescence intensity of the sulfo-Cy3 dye. The time-dependent increase in the fluorescence intensity of **DNA3** formed during the "photoclick" modification (Figure 1, bottom) tracks well with the observed differences in the absorption of **DNA1** (tetrazole at $\lambda_{max} =$ 355 nm) and **DNA3** (pyrazoline at $\lambda_{max} = 400$ nm).

To evaluate the scope of this "photoclick" modification, **DNA1** was irradiated in the presence of *N*-methylmaleimide, methylmethacrylate, and acrylonitrile. The first two reactions led to products **DNA4** and **DNA5**, respectively, in 42% and 23% yield, and identified by MALDI-TOF mass spectrometry (see the Supporting Information). The third reaction with acrylonitrile failed. The kinetic behavior of the bioorthogonal reaction is of significant interest with respect to its biological application. High rate constants are desirable, otherwise excess labeling reagent is needed, which could lead to solubility and toxicity problems.[12] We assumed a pseudo-first-order rate law; that means, the reaction rate is only dependent on the concentration of the dipolarophile **6**.[22] The reaction rates were determined on the basis of HPLC analysis of the yields of the reaction mixtures at two different concentrations of **6** (see the Supporting Information). The determined rate constant of $(23 \pm 7)\,M^{-1}s^{-1}$ is remarkably high with respect to other copper-free bioorthogonal reactions[12] and comparable with the copper-catalyzed cycloaddition between azides and acetylenes ($k \approx 10$–$200\,M^{-1}s^{-1}$).[12,13]

In conclusion we presented the photoinduced "click"-type postsynthetic modification of the oligonucleotide **DNA1** with the sulfo-Cy3 dye **6**. The corresponding synthetic DNA building block **5** carries the diaryltetrazole unit as the photoactivatable group attached to the 5-position of 2′-deoxyuridine through an aminopropynyl linker. This kind of attachment has two major advantages: Firstly, it potentially allows this tetrazole–nucleoside conjugate to be used in the enzymatic preparation of oligonucleotides (primer extension and PCR), since DNA polymerases (e.g. KlenTaq polymerase)[23] typically accept alkyne modifications in the 5-position. Secondly, the structural perturbation of the DNA by this kind of modification is expected to be rather small. The melting temperature of the modified **DNA1**–**2** is 55.2 °C, which is nearly identical to that of a completely unmodified DNA double strand with a T instead of the modified 2′-deoxyuridine (55.3 °C). The "photoclick" modification of **DNA1** can be performed by irradiation at 365 nm, outside the absorption range of nucleic acids, and by using LEDs as a cheap and reliable irradiation source. The reaction proceeds best in aqueous buffer solution and affords more of the desired **DNA3** if **DNA1**–**2** is applied as a double-stranded starting material. Both observations are ideal for application of this modification method in chemical biology, especially inside living cells. Moreover, it provides the advantages of a photochemical process, in particular spatial and temporal control.

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