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In situ azide formation and "click" reaction of nile red with DNA as an alternative postsynthetic route†

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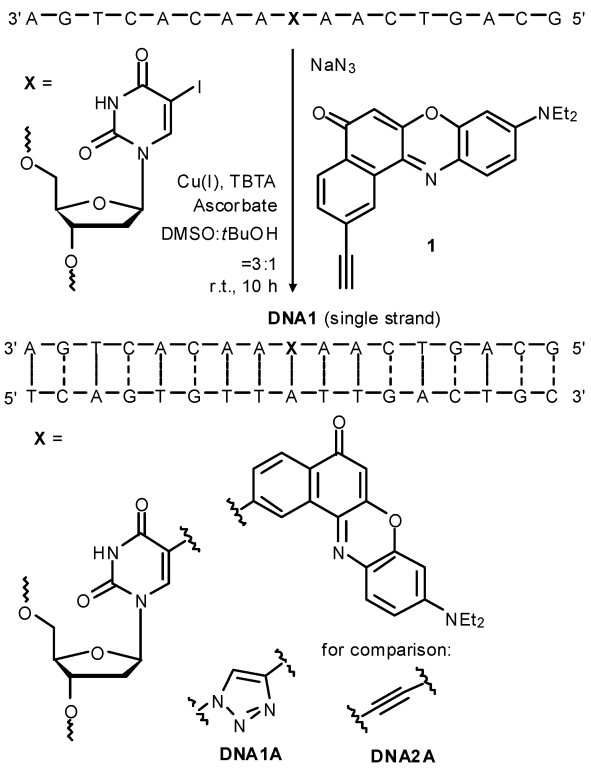
Oligonucleotides that contain a 5-iodo-2′-deoxyuridine in the sequence can be applied for a postsynthetic "click"-type modification with ethynyl-modified nile red as a fluorescent label by *in situ* formation of the intermediate azide.

The so-called postsynthetic modification of oligonucleotides allows the covalent attachment of molecules in a bioorthogonal fashion. This means that a special functional group of the modifier reacts specifically with a presynthesized oligonucleotide carrying the complementary functional group.[1] The time-consuming synthesis of phosphoramidites as DNA building blocks can be avoided which is especially important for precious or acid/base-labile probes or labels. The 1,3-dipolar cycloaddition that is based on Huisgen[2] and Sharpless[3] represents a ligation that matches the requirements of bioorthogonality since alkyne and azide typically are not present in oligonucleotides and react selectively with each other in aqueous solutions.[4] However, the published "click"-type modifications of oligonucleotides,[5,6] also inside cells,[7] are limited to reactions between acetylene groups as part of the oligonucleotide and azides of the modifying molecule but not *vice versa*. Although 5-azido-2′-deoxyuridine is known as a photoaffinity labeling reagent, it is generally assumed that azide-modified DNA bases exhibit chemical instability during automated DNA synthesis and workup.[8] We present here a first example of a postsynthetic method that allows the click reaction of an ethynyl-modified label (nile red) with an azide group in the 5-position of 2′-deoxyuridine which was formed *in situ* from a presynthesized oligonucleotide in one step. Furthermore, we compare the triazolyl bridge between the uracil moiety and the nile red dye with the previously published ethynyl bridge[9] by methods of optical spectroscopy.

This postsynthetic oligonucleotide modification is based on a solid phase protocol that we previously used for Pd-catalyzed Sonogashira-type couplings with ethynylpyrene.[10] The procedure starts with the synthesis of the oligonucleotide for **DNA1** bearing 5-iodo-2′-deoxyuridine in the middle of the sequence (Scheme 1). The corresponding phosphoramidite is commercially available. Subsequent treatment of the oligonucleotide (1 μmol) on solid phase with an excess of sodium azide (200 μmol in DMSO) yields *in situ* the 5-azido-oligonucleotide. In the presence of Cu(I) the ethynyl nile red **1** is then immediately coupled to the intermediate to give the nile red modified **DNA1**. Finally, the oligonucleotide is cleaved from the resin and deprotected under mild conditions (conc. NH_4OH, r.t, 18 h). The overall yield for the postsynthetic ligation to **DNA1** is 10% compared to the phosphoramidite building block approach that was used for **DNA2** in comparison.[9] The successful conjugation of nile red to the oligonucleotide has been verified by ESI mass spectrometry and optical spectroscopy. The duplex **DNA1A** was formed by heating **DNA1** in the presence of 1.2 equiv. of the unmodified counterstrand and subsequent slow cooling to r.t.

The UV/Vis absorption spectra of the single stranded **DNA1** and the duplex **DNA1A** show clearly the presence of the covalently attached nile red chromophore by a peak at *ca.* 610 nm (Fig. 1). When excited at 610 nm the steady state fluorescence exhibits a broad signal with a maximum at 658 nm and a quantum yield Φ_F of 29% in the single strand and 31% in the duplex. It is interesting to observe that the optical properties of **DNA1A** (Table 1) are very similar in comparison with **DNA2A** bearing the nile red dye attached



Scheme 1 Postsynthetic protocol for the preparation of the nile red modified **DNA1** and sequence of the corresponding duplex **DNA1A** in comparison to the previously published **DNA2A**.[9]

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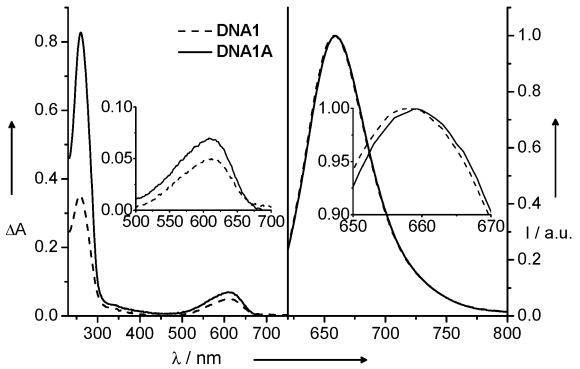

Fig. 1 UV/Vis absorption spectra (left) and fluorescence spectra (right) of single-stranded **DNA1** and the corresponding double-stranded **DNA1A**, each 2.5 μM in sodium phosphate buffer (10 mM) of pH 7.0, NaCl (250 mM), λ_{exc} = 610 nm.

Table 1 Comparison of the optical data of **DNA1A** with **DNA2A**[9] (T_m measured at 260 nm, 0.5 °C min^{-1}, 2.5 μM DNA in 10 mM Na–P_i buffer, pH 7, 250 mM NaCl).

	DNA1A	DNA2A[9]
λ_{max} (Absorption)/nm	612 (ss)	615 (ss)
	608 (ds)	615 (ds)
$\varepsilon/10^4$ $M^{-1}cm^{-1}$	2.0 (ss)	2.6 (ss)
	2.8 (ds)	2.5 (ds)
λ_{max} (Emission)/nm	658 (ss)	658 (ss)
	659 (ds)	665 (ds)
Φ_F[a]	0.29 (ss)	0.20 (ss)
	0.30 (ds)	0.10 (ds)
T_m/°C (ΔT_m/°C)[b]	58.1 (−2.6)	57.1 (−3.6)

[a] Determined with cresyl violet as the standard, ΦF = 0.54; [b] In comparison to a reference duplex with T instead X (T_m = 60.7 °C).

via an ethynyl linker to 2′-deoxyuridine (Scheme 1). Remarkably, the quantum yield of **DNA1A** is significantly higher than that of **DNA2A**. The melting temperature of **DNA1A** is 58.1 °C. This means that the attached nile red dye including the triazolyl linker destabilizes the duplex by 2.6 °C in comparison with the completely unmodified reference duplex. For **DNA2A** the thermal stability is slightly lower (57.1 °C)[9] indicating that the aromatic triazolyl bridge of the nile red conjugation in **DNA1A** is able to regain more of the lost thermal stability by aromatic interactions with the adjacent base pairs.

A critical issue for the optical properties of fluorophores is the linkage to the oligonucleotide. In this study, the nile red dye has been attached *via* the conjugating triazolyl bridge and compared with the ethynyl bridge. The LUMOs of both nile red modified nucleosides were calculated with the semiempirical AM1 method (Fig. 2). They were obtained after full geometry optimization. The results show clearly that both bridges lead to largely delocalized orbitals and to exciplex-like states that show unstructured, solvent-dependent fluorescent bands.

In conclusion, the described postsynthetic method completes the repertoire of "click"-type reactions for the

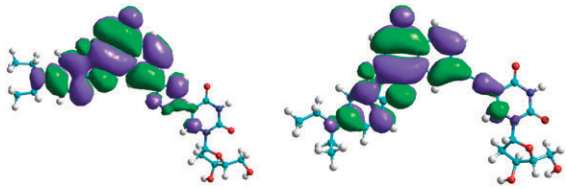

Fig. 2 Calculated LUMOs for the isolated nile red modified 2′-deoxyuridine of **DNA1A** (left) and **DNA2A** (right).

modification of oligonucleotides by providing a complementary access: in contrast to the conventional existing "click"-type modification procedures the acetylene group is provided as part of the modifier and reacts with an azido group that is formed *in situ* by treatment of a halogenated DNA base (5-iodo-2′-deoxyuridine) with sodium azide. This could be of significant advantage for the following reasons: (i) a lot of chromophores as labelling agents are typically provided with the ethynyl group since they were originally invented for functional π-systems. (ii) For the conventional modification of chromophores by an azide group a short alkyl linker between the aromatic system and the azide group is required due to the preparation by a nucleophilic substitution. However, the additional alkyl chain decouples the chromophore from the DNA base stack optically and electronically. (iii) Halogenated precursors of every DNA base are commercially available as DNA building blocks.[10] In our representative example, the optical properties (including the T_m value) of the modified DNA that contains the nile red conjugated to the 5-position of uridine *via* the triazolyl bridge are remarkably similar as in case of the ethynyl conjugation. These results underscore the significance of our synthetic approach.

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