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Bioconjugate Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.bioconjchem.5b00557 • Publication Date (Web): 20 Jan 2016

Polarity sensitive bioorthogonally applicable far-red emitting labels for postsynthetic nucleic acid labeling by copper-catalyzed and copper-free cycloaddition

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ABSTRACT

Two series of new, water soluble, membrane-permeable, far-red / NIR emitting benzothiazolium-based fluorescent labels with large Stokes' shifts were synthesized that can be conjugated to alkyne-modified biomolecules through their azide moiety via azide-alkyne cycloaddition. We have used these azide bearing labels to make fluorescent DNA constructs using copper-catalyzed "click" reaction. All dyes showed good or remarkable fluorescence intensity enhancement upon conjugation to DNAs. We also investigated the possibility to incorporate the benzocyclooctyne motif through rigid (ethnynyl) or flexible (ethyl) linkers into the DNA, thus enable copper-free labeling schemes. We observed that there is a marked difference between the two linkers applied in terms of optical properties of the labeled oligonucleotides. We have also tested the *in vivo* labeling potential of these newly synthesized dyes on HeLa cells previously transfected with cyclooctynylated DNA. Confocal fluorescent images showed that the dyes are all able to cross the membrane and suitable for background-fluorescence free fluorescent tagging of nucleic acids. Moreover, we have observed different accumulation of the two dye series in the endosomal particles, or in the nuclei, respectively.

INTRODUCTION

Molecular biology methods aiming at observing and studying biochemical processes or biomolecules in their natural environment have benefited greatly from fluorescence-based imaging techniques. Detection of the emitted light offers a highly sensitive and relatively cheap means of tracking with excellent temporal/spatial resolution.[1,2] The use of various fluorescent labels in combination with other detection channels further increases the range of imaging methods.[3,4] There are several ways to selectively tag the ensued biological entities: fluorescently labeled antibodies usually offer highly selective and sensitive staining of the biomolecule of interest, however, due to the considerable size of antibodies their use is limited to extracellular structures or fixed cells.[1] Genetically encodable fluorescent proteins (FPs) can, in theory, be used to tag any protein of interest in various colors. Multi-color FP-based molecular biology techniques that emerged in the last decades are well credited as they opened a new era in molecular biology related imaging techniques.[1,5] However, the use of FPs is limited to the labeling of biomolecules that are directly encoded in the genome i.e. proteins. Post-translational modifications of proteins is also hard to follow with FPs, although recent efforts are quite promising in this regard.[6] Besides, the larger size of GFP or its variants often influences the native function of the proteins studied. A third way for the selective installation of fluorescent tags onto biomolecules became possible with the introduction of bioorthogonal manipulation methods.[7,8,9,10] These chemical reactions between non-native functions ensure exclusive relationship between appropriately modified biomolecules and labels. Parallel evolution of genetic code expansion techniques enabled the researchers to selectively modify virtually any proteins with bioorthogonalized, non-canonical amino acids.[11,12] Moreover, bioorthogonal labeling is not limited to proteins. By means of metabolic incorporation of non-natural building blocks (nucleotides, lipids, carbohydrates) or chemical targeting of unique motifs (rare amino acids, or short, specific tags), enables labeling of biopolymers, lipids and small metabolites as well.[13,14,15] Bioorthogonal labeling schemes usually follow a sequential, two-step routine. Implementation of the first bioorthogonal function into the biomolecule of interest is followed by fluorescent tagging with a small, synthetic label harboring the complementary cognate function.[16,17] Photophysical properties, reactivity, membrane-permeability of these small labels can be synthetically tailored. Most biomolecular tagging by bioorthogonal means deal with the fluorescent modification of proteins. This is mainly due to the profound biological importance of this group, but also due to genetic code expansion based advanced technologies. Other biomolecules were also manipulated bioorthogonally

with fluorescent labels, yet, bioorthogonal modification of other biomolecules remains somewhat less investigated. Especially bioorthogonal labeling for fluorescent nucleic acid modifications, and corresponding applications with DNA and RNA in living cells have only been rarely realized[18] since this *in-vivo*-chemistry is just developing.[19] This must be a major goal for the actual research as it will provide the important chemical-biological impact for fluorescent nucleic acid probes.

Detection of a specific fluorescent signal is usually compromised by the appearance of non-specific signals when using small synthetic labels. On the one hand autofluorescence of naturally occurring chromophores can be circumvented by the use of far-red, near infrared (NIR) emitting fluorophores with large Stokes' shift.[5] On the other hand, to diminish background fluorescence caused by non-specific sticking of labels to non-polar surfaces is more challenging. One solution to overcome background fluorescence is to use so-called fluorogenic tags, whose fluorescence is turned on upon the specific chemical transformation during conjugation.[20,21,22,23] We have recently demonstrated that such probes are suitable to diminish background fluorescence.[22,23] Although some methods exist that can facilitate the design of such fluorogenic tags by theoretical prediction methods, the real behavior of such fluorogens, is rather empirical. There is another option that mimics chemically triggered fluorogenicity. That is to use fluorescent labels with very low quantum yields, whose fluorescence is largely dependent on the microenvironment.[24,25] Recently, we have reported on the synthesis and polarity dependent behavior of a set of NIR emitting dimethine dyes with large Stokes' shifts in connection with nucleic acid labeling.[26] Following these observations we have designed a new set of polarity sensitive labels and investigated their applicability in nucleic acid labeling studies.

RESULTS AND DISCUSSION

In the design process we aimed at developing bioorthogonally conjugatable, polarity sensitive labels with polar characteristics in order to minimize the possibility of non-specific sticking to hydrophobic surfaces. As a starting point, we used frameworks (Figure 1) that we formerly found amenable of conjugation mediated fluorescence turn on.[26,27] It was hypothesized that replacement of the lepidinium core of dyes **1a,b** with more polar ethyl-benzothiazolium unit (**2a**, **2b**) would substantially increase polarity.

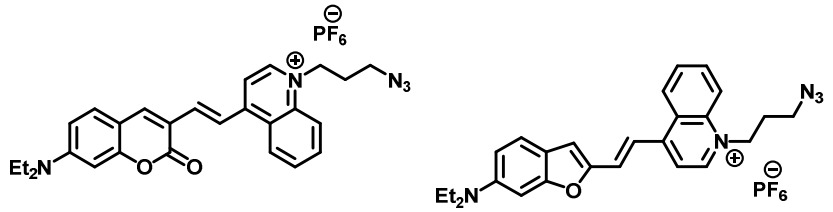


Figure 1. Bioorthogonally applicable, NIR emitting labels with large Stokes' shifts.[26,27]

Furthermore, in order to increase membrane-permeability for future applications we also aimed at investigating the effect of a more polar a propylsulfonyl pendant (**3a**, **3b**). These latter dyes are zwitterionic, with a net 0 charge, features that most membrane-permeable dyes share. Thus, we set forth synthetic routes in order to access labels **2** and **3** (Figure 2). It was also hoped, that this latter modification will shift excitation and emission maxima towards the far-red end or NIR regime of the spectrum.

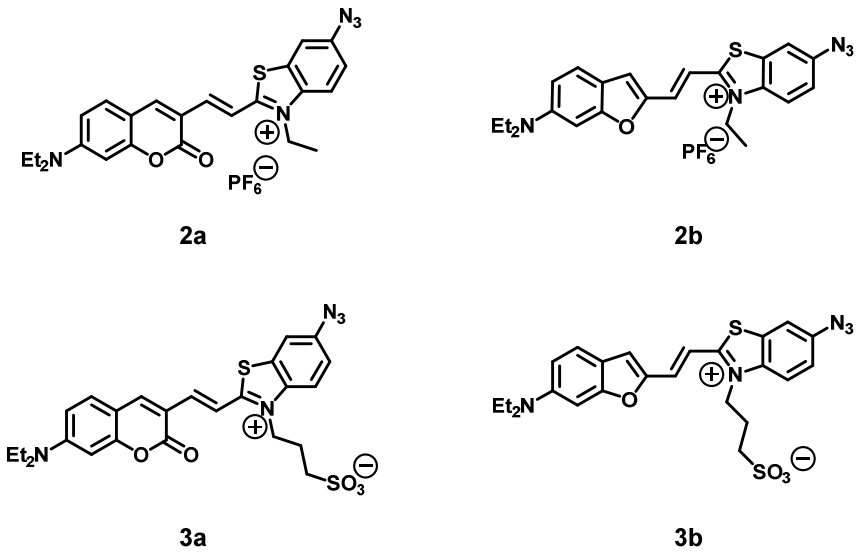
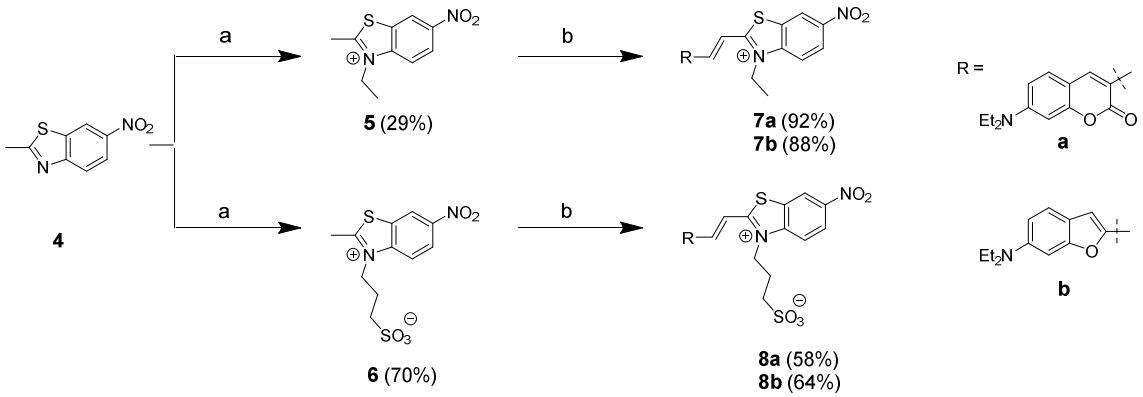


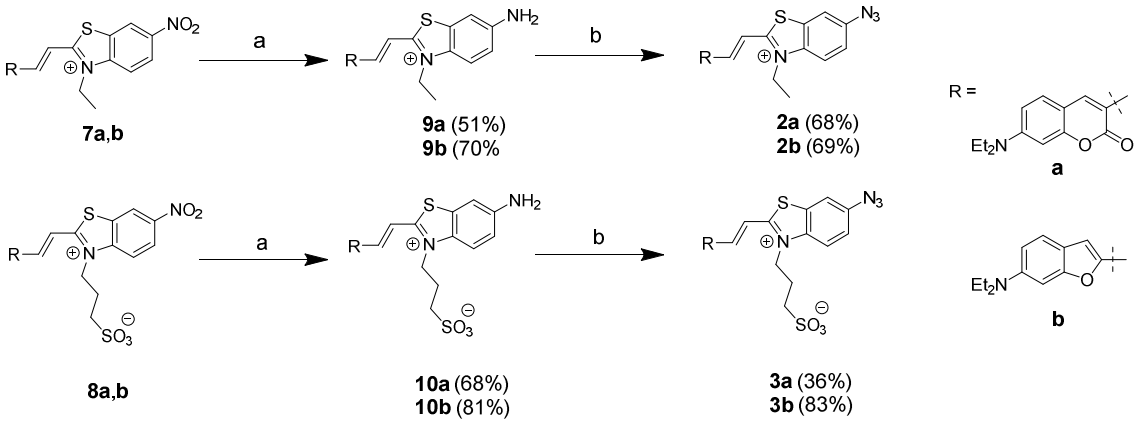
Figure 2. Proposed target structures **2a**-**3b**.

We have started with 2-methyl-6-nitrobenzo[*d*]thiazole that was obtained from commercially available 2-methylbenzo[*d*]thiazole upon standard nitration reaction. Alkylation of **4** with ethyl iodide or 1,3-propanesultone afforded compounds **5**, **6** in medium to good yields. Base catalyzed condensation of **5** and **6** with 3-formylcoumarin or 2-formylbenzofuran yielded **7** and **8** (Scheme 1).



Scheme1. Synthesis of nitro-precursors **7** and **8**. a) EtI, 190 °C, 20 min or 1,3-propanesultone, 100 °C, 30 min; b) 3-formylcoumarin or 2-formylbenzofuran, piperidine (cat.), EtOH, reflux, 5 h.

Reduction of the nitro group was effected by treatment with tin-chloride to allow the corresponding amines **9** and **10**. All amines were subjected to a standard diazotization/azidation sequence using sodium nitrite and sodium azide to furnish target compounds **2** and **3** in good yields (Scheme 2). Compounds **2a**, **b** were converted to their respective hexafluorophosphate salts by treatment with excessive amount of NH_4PF_6 and subsequent precipitation from aqueous solutions.



Scheme 2. Synthesis of target compounds **2**, **3**. a) $SnCl_2.2H_2O$, $_{cc}HCl$, 22 °C, 3 or 5 h; b) $NaNO_2$/HCl, 22 °C, 15 min then NaN_3, in 1:1 HCl, 22°C, 1.5 h or 4.5 h

Since in biomolecular conjugation schemes the azide moiety is to be converted to the corresponding triazole derivative we have made the respective model triazoles (**11** and **12**) in Cu(I) catalyzed azide-alkyne cycloaddition reactions with *N*-(prop-2-yn-1-yl)pivalamide.

In order to assess the spectroscopic characteristics the azides and triazoles we have acquired absorption, emission and excitation spectra and determined molar absorptivities and quantum yields for each compound.

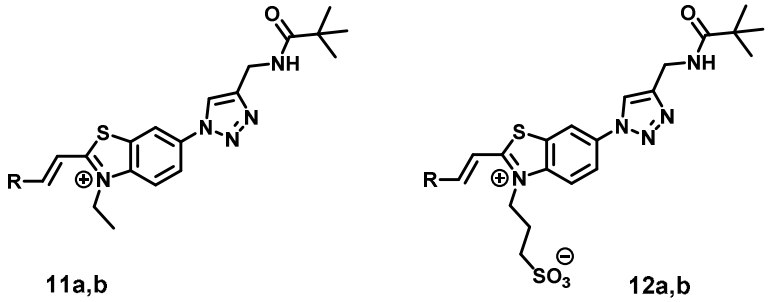


Figure 3. Model triazoles **11**, **12**.

As expected, all dyes and their triazolic congeners were excitable in the visible region of the spectrum with emission maxima in the far-red, near infrared region with notably large Stokes' shifts. Water solubility of the dyes improved remarkably with respect to their lepidinium-based predecessors. Further photophysical characterization of the azides and their triazolic products revealed that the brightness of the respective triazoles is lower than that of the parent azides. This was mainly attributed to the notably lower molar absorptivities of the triazoles, while quantum efficiencies remained practically unchanged (Table 1).

Table 1. Summary of optical properties, of azides and triazoles **2-3** and **11-12**

	Compounds							
	2a	**11a**	**3a**	**12a**	**2b**	**11b**	**3b**	**12b**
$\lambda_{abs\ PBS}$ [nm]	543	550	548	567	579	612	591	598
$\varepsilon_{abs\ PBS}$ [$\times 10^4$ $M^{-1}cm^{-1}$]	6.86	1.96	4.52	1.54	4.00	2.99	3.97	1.02
$\lambda_{exc\ PBS}$ [nm]	533	556	550	556	606	604	600	593
$\lambda_{em\ PBS}$ [nm]	656	664	653	655	718	718	715	714
ϕ (%)[a]	6.36	5.07	5.32	5.32	1.7	1.37	1.64	1.19
$\lambda_{abs\ DMSO}$ [nm]	563	579	565	567	604	616	606	613
$\varepsilon_{abs\ DMSO}$ [$\times 10^4$ $M^{-1}cm^{-1}$]	6.59	1.65	3.85	1.57	5.13	2.93	4.00	1.17
$\lambda_{exc\ DMSO}$ [nm]	558	566	556	560	604	604	602	610
$\lambda_{em\ DMSO}$ [nm]	661	667	676	682	724	729	729	731
ϕ (%)[a]	32.0	36.0	27.0	28.0	9.7	9.7	10.1	9.7

[a] relative to Rhodamine 101 and cresyl violet for the **a** and **b** series, respectively

In order to evaluate the fluorescence properties of the newly synthesized dyes in conjugates we attached them covalently to DNA single and double strands. The oligonucleotides were automatically synthesized using standard conditions on solid phase. Dyes **2a-3b** were postsynthetically incorporated according to our published protocol using copper(I)-catalyzed

cycloaddition.[28] The bioorthogonally reactive DNA building block of 2'-propargyl-modified uridine is commercially available. The resulting single-stranded **DNA1a-DNA3b** were identified by MALDI mass spectrometry and quantified by UV/vis absorption spectroscopy. The double stranded **DNA4-DNA9** were annealed using the corresponding unmodified counterstrands by heating to 90 °C for 10 min, followed by slow cooling to room temperature.

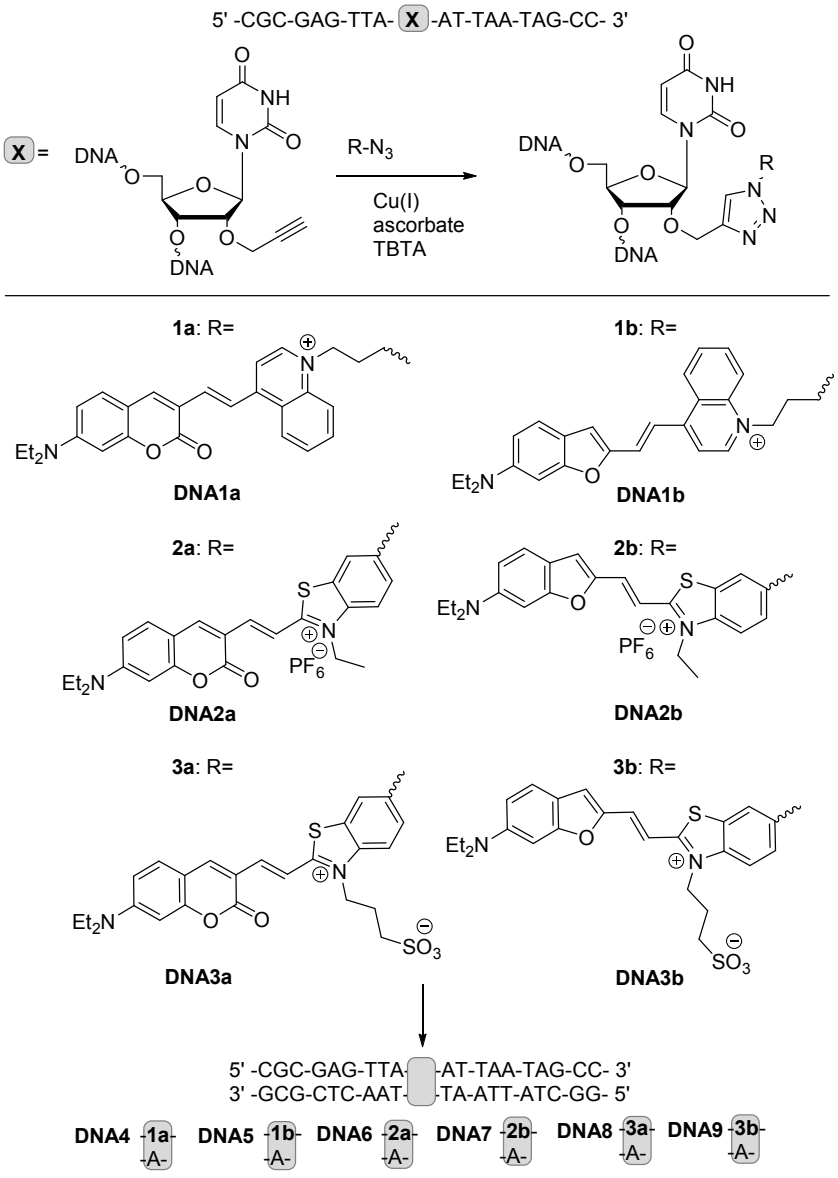


Figure 4. Sequences of **DNA1a-DNA9** and structure of 2'-modifications.

As mentioned before the background fluorescence of the dyes without DNA is very low. By contrast, covalent linkage of the bioorthogonally reactive dye azides to DNA resulted in remarkable fluorescence intensity increase at maxima of the triazolic products. Figure 5 representatively shows the difference between the emission intensity of the dye **2a** without DNA, and as part of single-stranded **DNA2a** and double-stranded **DNA6**. This observation is typical for cyanine-styryl dyes. In solution without interaction with DNA, the two heterocycles have the possibility to rotate around their methine bridge giving rise to non-radiative relaxation pathways. In the case of covalent linkage to DNA this radiationless pathways to dissipate excitation energy gets lost and fluorescence becomes the primary relaxation process. These interactions slightly change from single to double strands as the absorption maximum shifts from 591 nm to 578 nm and the emission maximum shifts from 651 nm to 640 nm. Concomitantly, the quantum yield increases further from 55% in the single strand to 70% upon anellation with the counterstrand.

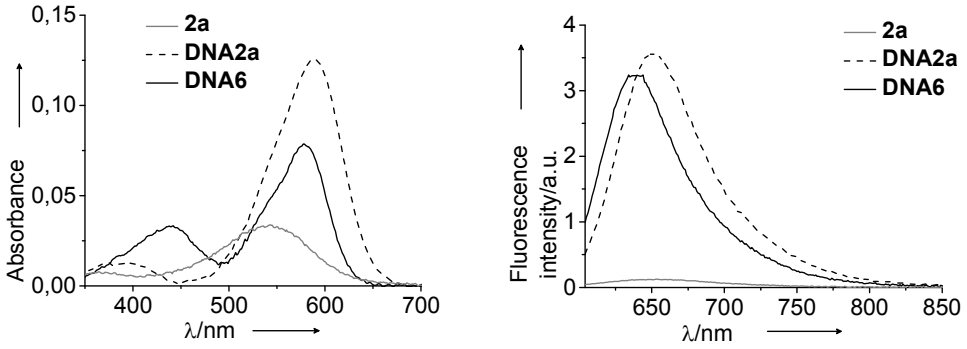


Figure 5. Comparison of the absorption (left) and fluorescence (right) of **DNA2a**, **DNA6** and dye **2a**

Table 2 summarizes all optical properties of the synthesized dye-oligonucleotide conjugates. The quantum yields of the single-stranded **DNA2b** and **DNA3b** and corresponding double strands **DNA7** and **DNA9** are comparable. Obviously, the influence of the negative charge on dye **3b** is negligible. Contrary to this, incorporation of coumarin derivatives **2a** and **3a** into the nucleic acid strands yields a significant increase of the quantum yield upon anellation to up to 70% in **DNA6**. There seems to be a major change of interactions between DNA and the covalently attached dye associated to the coumarin motif since dyes **2a** and **3a** induce destabilization of the corresponding DNA double strands by 4 °C whereas dyes **2b** and **3b** yield a stabilization by 6 °C, each compared to unmodified DNA double strand as reference.

Table 2. Summary of optical properties, quantum yield (ϕ_F) and melting temperatures of **DNA1a-DNA13**.

DNA	$\lambda_{max,abs}$ [nm]	$\lambda_{max,em}$ [nm]	ϕ_F [%]	T_m [°C]
DNA1a	585	703	16.0	-
DNA1b	649	792	1.8	-
DNA2a	591	651	55.1	-
DNA2b	669	731	8.1	-
DNA3a	597	657	42.3	-
DNA3b	679	737	8.0	-
DNA4	562	695	17.5	67
DNA5	644	795	0.7	66
DNA6	578	640	70.3	60
DNA7	646	721	14.4	71
DNA8	585	646	58.0	61
DNA9	659	725	13.0	71
DNA10b	612	730	8.7	-
DNA11b	658	730	8.6	-
DNA12	657	722	9.4	54
DNA13	654	721	13.2	53

Despite the broad applicability of the copper(I)-mediated cycloaddition chemistry between azides and ethynyl groups, as demonstrated above, the use of metal catalysts, especially the cytotoxic copper ions, bears significant risks for live cell imaging. Extensive purification by HPLC or the use of additives (such as EDTA) is needed to provide real copper-free fluorescent DNA probes. Among the copper-free alternatives[19], the strain-promoted cycloaddition between cyclooctynes and azides represents an important alternative since it allows a catalyst-free means of labeling. For this purpose, we recently established the carboxymethylmonobenzocyclooctyne (COMBO)[29] group as a structurally simple, non-fluorinated copper-free "click" reagent that offers easy access to bioconjugates through its carboxy function and exhibits lower lipophilicity in comparison to its doubly benzofused alternatives.[29] Moreover, COMBO exhibits good chemical stability, thus enable the preparation and use of a new DNA building block with the COMBO group attached to the 5-position of a 2'-deoxyuridine in DNA allowing rapid and efficient copper-free postsynthetic

modification with azido-dyes.[26] The fast and efficient labeling potential of COMBO was also recently demonstrated by us in combination with tetrazine-tagged nucleic acids.[30] We applied this building block for representative labelling with fluorescent dye **3b** in order to track down any effects that might affect fluorescence properties that are associated with the kind of linkage to the DNA. Accordingly, oligonucleotide **DNA10b** and its corresponding double stranded **DNA12** were synthesized. The fluorescence quantum yield of this DNA double strand is unspectedly low (9%) which was attributed to the rather rigid and inflexible ethynyl linker between the 2'-deoxyuridine part and the fluorophore which interferes with best possible interactions of the dye with the DNA. Hence, a new DNA building block **13** that also carries the COMBO group attached to the 5-position of 2'-deoxyuridine but linked via a saturated alkyl and thereby a flexible linker (Figure 6) was synthesized.[31,32,33,34]

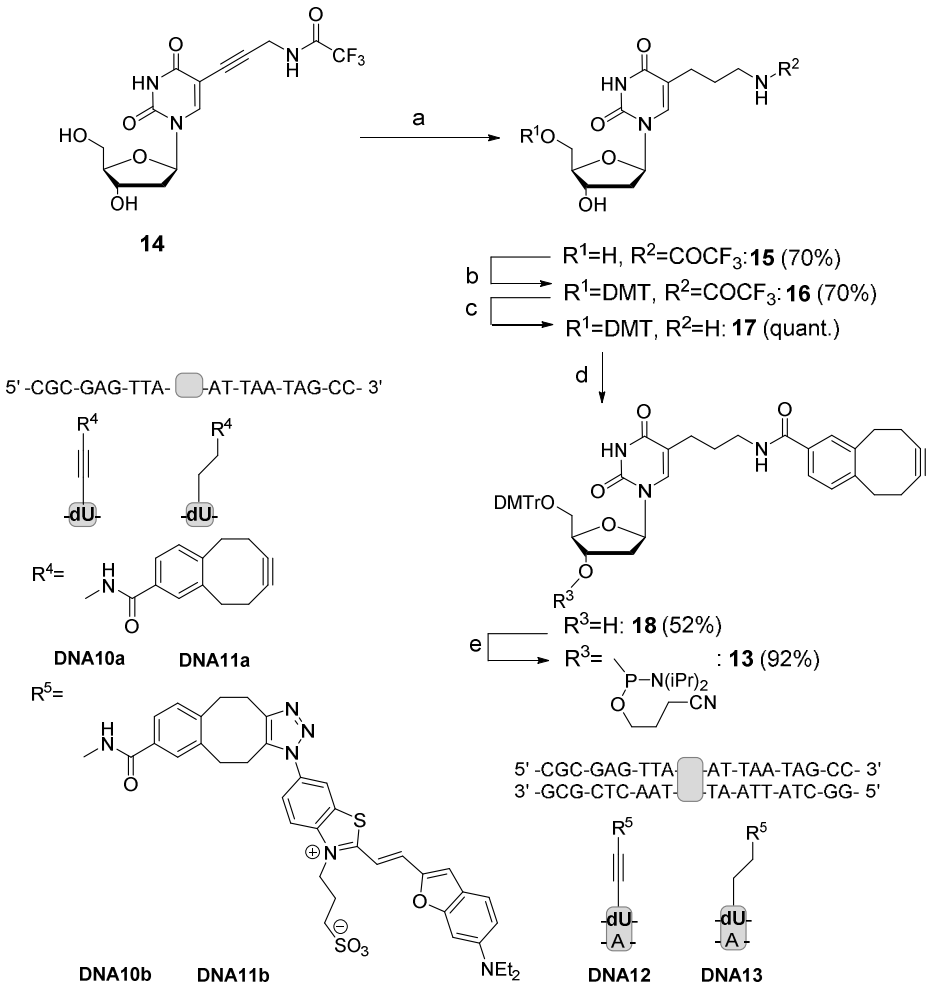


Figure 6. Synthesis of the phosphoramidite **13** as new COMBO-modified DNA building block, and sequences of **DNA8** and **DNA9**. a) 20% $Pd(OH)_2/C$, $HSiEt_3$, MeOH, rt, 24 h; b) DMTCl, Et_3N, pyridine, 40 °C, 18 h; c) NH_4OH; d) COMBO, HBtU, $HOBt \cdot H_2O$, DMF, DIPEA, rt, 2 h; e)

$Cl(iPr)_2NPO(CH_2)_2CN$, DIPEA, CH_2Cl_2, rt, 1 h. The corresponding single strands are named **DNA10b** and **DNA11b** for double stranded **DNA12** and **DNA13**, respectively.

The synthesis applies the trifluoroacetyl-protected 5-(3''-aminopropynyl)-2'-deoxyuridine **14** as starting material that can be obtained following literature procedures.[34] The key step is the hydrogenation of the ethynyl linker that can be achieved by treatment with $HSiEt_3$ using 20% $Pd(OH)_2/C$ as catalyst.[35] The new and saturated nucleoside **15** is obtained in 70% yield. The subsequent steps are very similar to the previously published procedure for the COMBO-modified DNA building block with the ethynyl linker.[26] First, the DMT group is introduced to the 5'-position, then the TFA protecting group is removed from the amine, which allows subsequently to couple COMBO using a peptide coupling procedure (HBtU/HOBt). Finally, the introduction of the phosphoramidite group to the 3'-position completes the synthesis of **13**. The oligonucleotide **DNA11b** was synthesized on solid phase using automated standard conditions, including workup, purification by HPLC and identification by MALDI-TOF mass spectrometry. The correspondingly modified double strand **DNA13** shows, as expected, a significantly increased fluorescence quantum yield. In fact, the quantum yield of **DNA13** is nearly identical to that of **DNA9**.

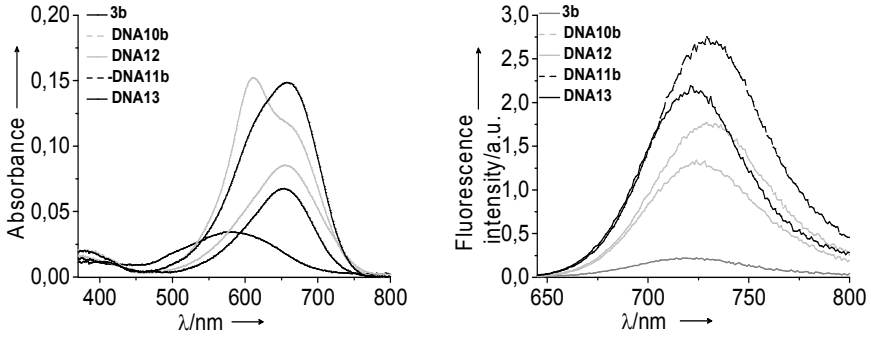


Figure 7. Comparison of the absorption (left) and fluorescence (right) of **3b**, **DNA10b**, **DNA11b**, **DNA12** and **DNA13**.

Finally, we performed live cell experiments in order to demonstrate the potential of the newly synthesized dyes in fluorescent imaging. Prior to treatment with the corresponding azido-dyes, 5x 10^4 HeLa cells were transiently transfected with a 15 pmol single stranded DNA that was modified with COMBO through the flexible linker (**DNA11a**), and Screenfect®, for 24 hours. The **DNA11a** transfected cells were then treated with 30 pmol of dyes **1a**-**3b** for 24 hours, then imaged by confocal fluorescent microscopy (Figure 8). In comparison to non-transfected control cells specific fluorescent staining could be observed, indicating that all dyes tested are taken up by the cells irrespective of having +1 or zero net charge. This is in

accordance with literature examples (e.g. Rhodamine-dyes or ATTO-dyes).[36] Contrary to these previous examples, however, the dyes tested in this work show no measurable non-specific background fluorescence that could originate from adsorption to hydrophobic, and/or negatively charged surfaces (e.g. DNA). The fluorescence images reflect the quantum yield differences between the dyes. The dyes show different accumulation preferences depending on their cellular uptake mechanism. Remarkably, coumarin derivatives (**2a**, **3a**) showed specific DNA staining in the nuclei, while benzofurane- tagged DNA molecules (**2b**, **3b**) were accumulated preferably in endosomal vesicles. MTT assays revealed that none of the dyes are toxic at the concentration levels used for this labeling experiments (0.15 μM) (SI Figure 17-22). Therefore, all dyes are well suited for *in vivo* click reactions.

We also compared these new dyes with our previously reported lepidinium-dyes, (**1a**,**b**). Dye **1b** showed fluorescence intensity that so low, that the results are hard to interpret. On the other hand, **1a** exhibited similar features to the newly synthesized series. We also noticed that dyes **2**-**3** are much more soluble in aqueous media than lepidinium dyes **1**. Overall, these results show clearly, that the newly prepared dyes have a significant potential for low-background live cell imaging schemes, especially those that bear higher quantum yields.

CONCLUSIONS

In conclusion, we have synthesized a set of new benzothiazolium containing dimethine dyes harboring an azide function enabling bioorthogonal conjugation of the dyes to alkyne functionalized targets. Photophysical characterization of the respective triazolic products revealed that they are all excitable in the visible regime of the spectrum with emission maxima in the far-red, near infrared region. Water solubility of the dyes improved remarkably with respect to their lepidinium-based predecessors and large Stokes' shifts were observed in each case. As expected the fluorescent quantum yields were low in aqueous solutions, however, in each case, marked fluorescence enhancements were observed when the dyes were conjugated to DNA targets. This effect was much more pronounced for the **a** series of the dyes, suggesting that the coumarin moiety interacts with the DNA bases in a different manner than the benzofurane (series **b**) at the expense of stabilization of the double strand. Furthermore, we have investigated the possibility of cyclooctyne motif incorporation into the oligonucleotide strands in order to enable copper-free labeling protocols. Of the linkers through which the cyclooctyne is attached to the DNA base we found that the more flexible

linker allows similar interactions between the dyes and DNA, which realized in nearly identical enhancement of fluorescence upon copper-free conjugation of dye **3b** to **DNA13** as observed in the case of copper-catalyzed construction of **DNA9** conjugate. We also tested the *in vivo* applicability of these new azido-dyes on HeLa cells, transfected previously with cyclooctynylated-DNA (**DNA11a**). All dyes were taken up by the cells with no observed toxicity and could specifically tag the target DNA. Non-transfected cells on the other hand showed no appreciable fluorescence indicating that these dyes are suitable for background-fluorescence free imaging of alkyne modified biomolecular targets using far-red light.

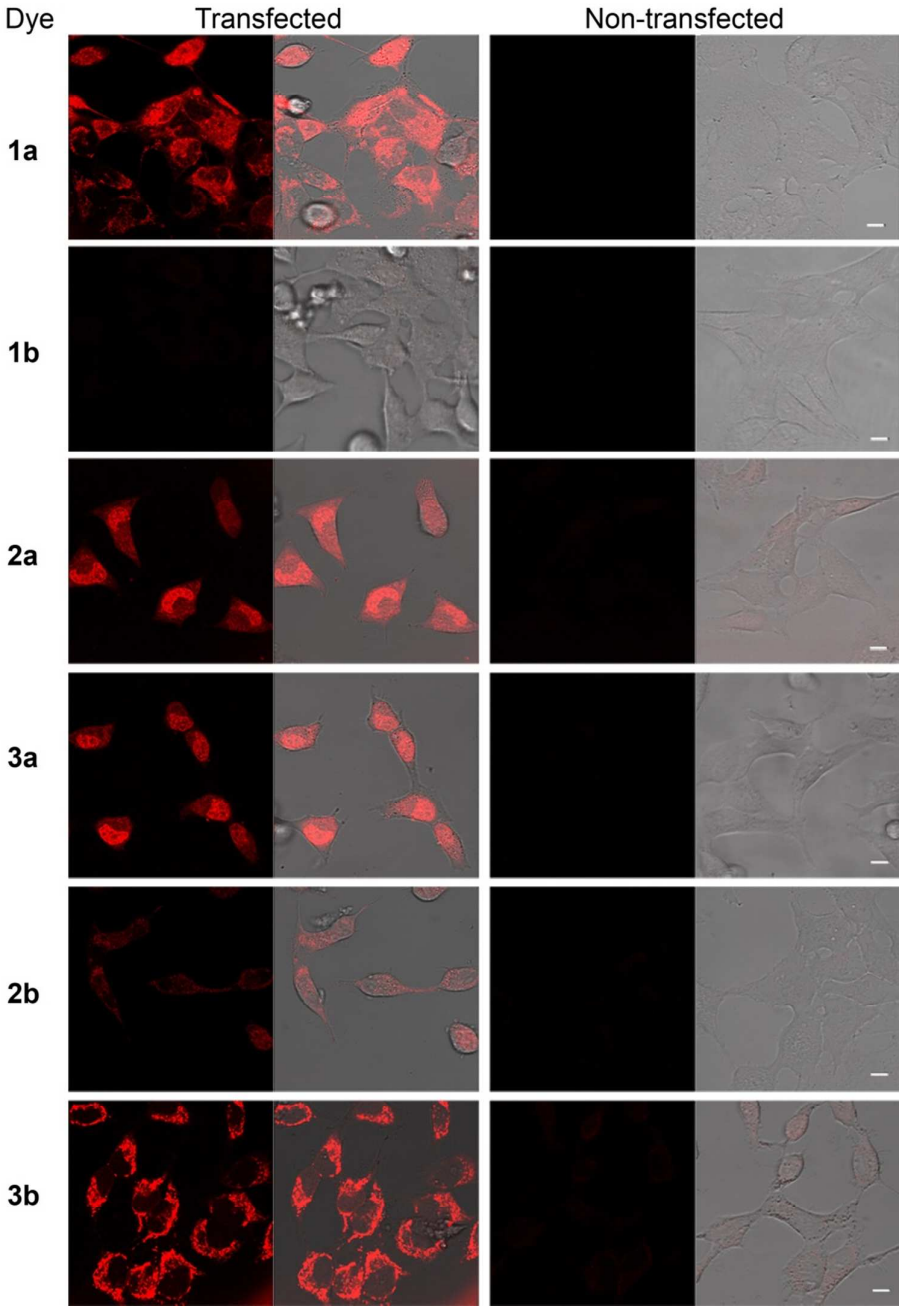


Figure 8. In vivo click reaction of **DNA11a** and dyes **1a-3b**; 1x 10^4 Hela cells are transfected with **DNA11a** and subsequently incubated with dyes **1a-3b**. Left column fluorescence confocal image, right column: Merge of fluorescence image with bright field. Scale bar 10 μm.

ACKNOWLEDGEMENTS

Present 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) and by the Deutsche Forschungsgemeinschaft (grant Wa 1386/16-1) and by the GRK 2039 (funded by DFG) and by the Helmholtz Program "Biointerfaces in Technology and Medicine" (BIF-TM).

Supporting Information Available:

Details of synthetic procedures, characterization data and spectra are available. Details of DNA labeling experiments and toxicity tests are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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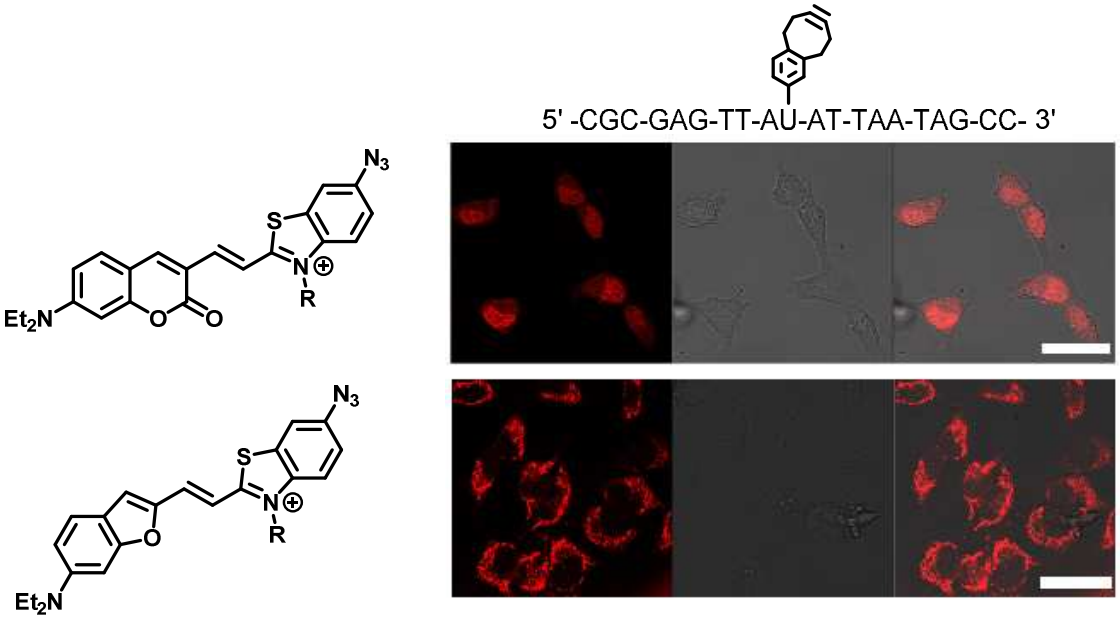
Graphical abstract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