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Two-Factor Fluorogenicity of Tetrazine-Modified Cyanine-Styryl Dyes for Bioorthogonal Labelling of DNA

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Abstract: Two green fluorescent tetrazine-modified cyanine-styryl dyes were synthesized for bioorthogonal labelling of DNA by means of the Diels-Alder reaction with inverse electron demand. With DNA as target biopolymer the fluorescence of these dyes is released by two factors: (i) sterically by their interaction with DNA, and (ii) structurally via the conjugated tetrazine as quencher moiety. As a result, the reaction with bicyclononyne-modified DNA is significantly accelerated up to $\geq$284,000 $M^{-1}s^{-1}$, and the fluorescence turn-on is enhanced up to 560 by the two-factor fluorogenicity. These dyes are cell permeable even in low concentrations and undergo fluorogenic reactions with BCN-modified DNA in living HeLa cells. The two-factor fluorescence release improves the signal-to-noise ratio such that washing procedures prior to cell imaging are not needed, which is a great advantage for live cell imaging of DNA and RNA in the future.

Introduction

Since first mentioned in 1893 by Pinner et al.,[1] tetrazines have been used in numerous applications ranging from materials,[2,3] total synthesis,[4–6] coordination chemistry,[7–9] to functional groups for inverse electron-demand Diels-Alder reactions (iEDDA).[10–12] These reactions belong to the fastest "click" reactions currently available.[13] This type of bioorthogonal reaction does not require the use of cytotoxic metal catalysts, light or additives, and is therefore a highly useful synthetic tool in chemical biology. Initially, tetrazines for cell imaging were attached to dyes via simple alkyl linkers.[14] In these cases, tetrazines were electronically decoupled from the fluorophore and applied solely as reactive dienes to attach dyes rapidly and efficiently to biomolecules, like the bicyclononyne (BCN)-GFP-conjugated protein.[15] The fluorescence quenching capabilities of tetrazines were exploited independently by Weissleder et al. and Wombacher et al. for rhodamines and BODIPYs.[16,17] The fluorescence is restored solely by the conversion of the tetrazine as quencher into the non-quenching diazine during the iEDDA reaction with proteins. Devaraj et al. used this concept for nucleic acid-templated ligations and phospholipids.[18] The tetrazine quenching concept was applied just once for nucleic acid labelling in living cells (not fixed cells) by means of a different strategy.[19]

Additional to fluorogenicity, dye photostability is a challenge for live cell imaging.[20] Our cyanine-styryl dyes show optimized photostability albeit they are relatively small. They are easy to synthesize by their modules.[21] They exhibit large Stokes shifts, which separates the excitation light from the emission.[22] Most importantly, they serve as light-up probes for DNA, similarly to thiazole orange and derivatives, like SYBR Green.[23,24] The interaction with DNA blocks depopulation pathways from the excited state which quenches their fluorescence. Such pathways are initiated by the intramolecular twisting around the central bridge bonds between the two aromatic systems of the dyes which are turned down when these dyes are bound to DNA. By conjugating cyanine-styryl dyes to tetrazines, it should be possible to achieve a two-factor fluorogenicity for these dyes with DNA as target biopolymer: sterically when bound to DNA, and structurally via the electronically coupled tetrazine as quencher moiety (Figure 1).[25] In this work, we present the two green fluorescent tetrazine-modified cyanine-styryl dyes **3a** and **3b** for bioorthogonal labelling of DNA. These dyes combine both fluorescence release mechanisms and thereby the two-factor fluorogenicity. Experiments in living HeLa cells demonstrate that this "click" reaction is compatible with the cellular environment and does not require washing steps to image the labelled DNA.

Results and Discussion

Methylated tetrazines are often preferred over unsubstituted (H-)tetrazines because they represent one of the best compromises between stability and reactivity.[26] We conjugated methyltetrazines to two different cyanine-styryl dyes via ethenyl linkers for conjugation to ensure efficient fluorescence quench-

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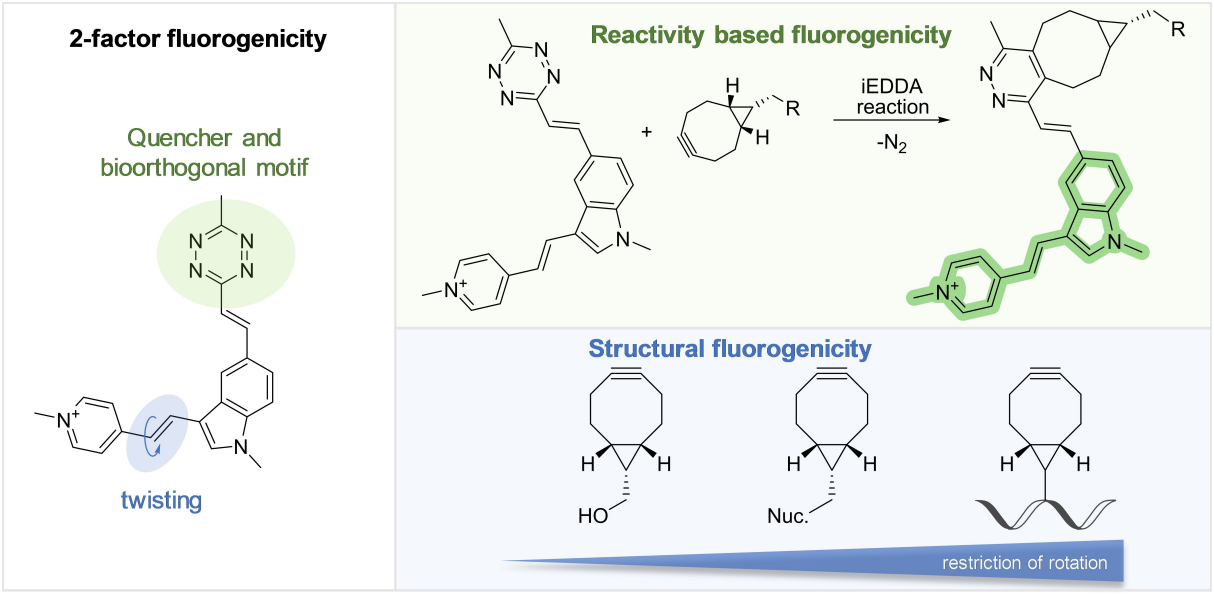
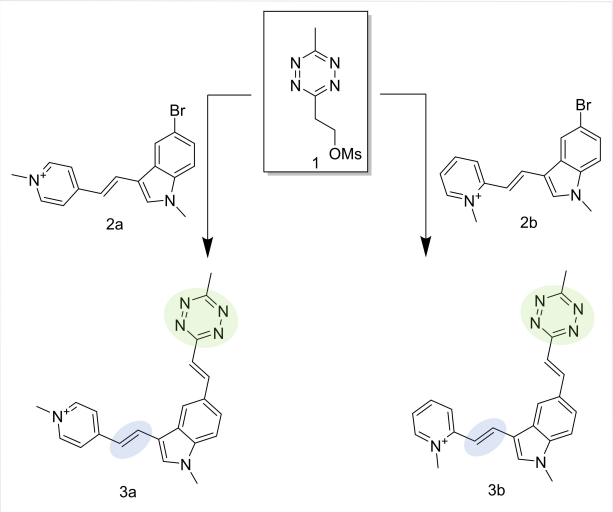


Figure 1. Two-factor fluorogenicity of tetrazine-modified cyanine-styryl dyes. The tetrazine serves as bioorthogonally reactive group and fluorescence quencher at the same time. (i) An increase of fluorescence intensity is achieved upon conversion to the diazine by the iEDDA reaction. (ii) Nucleoside and DNA binding results in structural fluorogenicity.

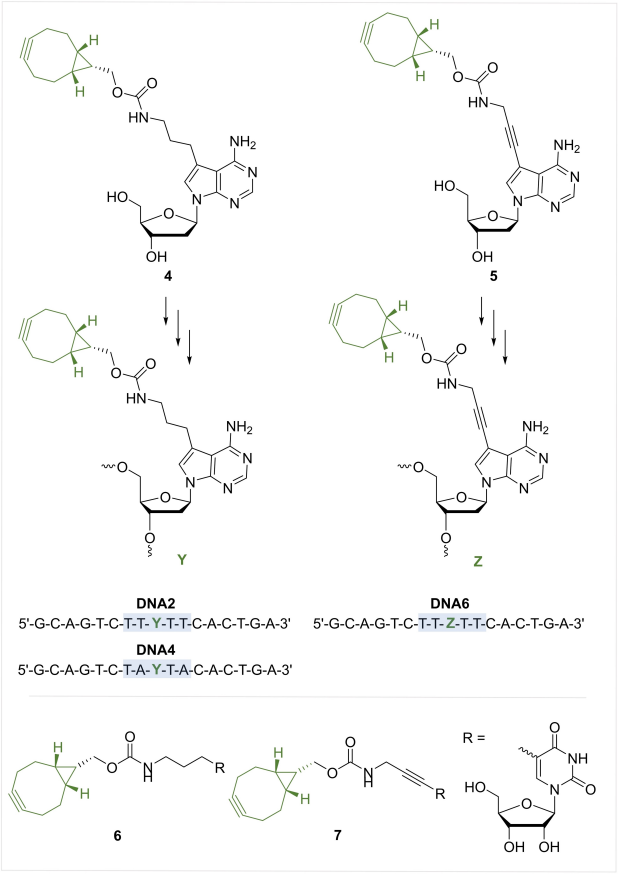
ing. The synthesis of the dyes **3a** and **3b** followed our modular approach (Schemes 1 and 3, Figures S48–S71).[22] We used 5-bromoindole as starting material to synthesize the dye precursors **2a** and **2b** in a simple 3-step procedure. This includes a Vilsmeier-Haack formylation at the position 3 of the indole, followed by a methylation of the amine functionality at the indole using dimethyl carbonate and K_2CO_3, and finally the condensation with the dimethylpyridinium cation in excellent overall yields of 86% over all three steps. We found that this procedure is easily scalable up to ten grams and delivers the products of very high purity without the need for extensive purification steps. The methyltetrazine building block **1** was synthesized in sufficient yields with 3-hydroxypropionitrile as starting material.[10] The precursors were conjugated to the desired dyes **3a** and **3b** by adapting a method from Devaraj et al.[10] We omitted the microwave irradiation and heated the substrate solutions in crimp vials in a metal heating block under an argon atmosphere. We obtained the pure products with yields of 33% in both cases.



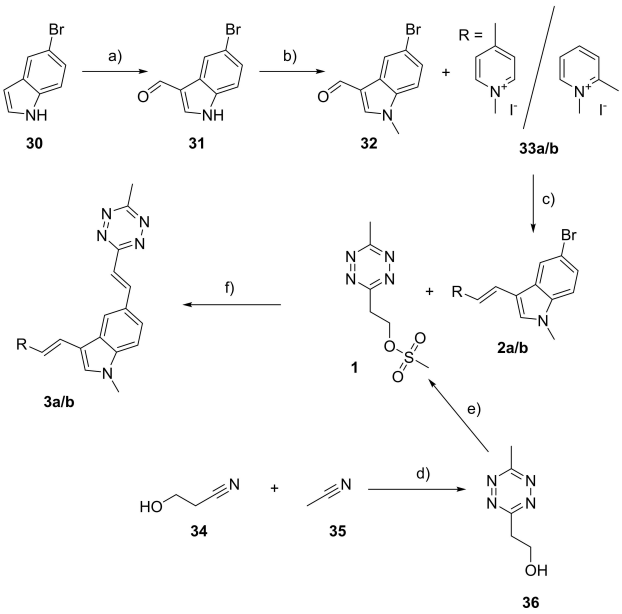
Scheme 1. Synthesis of the dyes **3a** and **3b** via Heck coupling of the precursors **2a** and **2b** with tetrazine precursor **1**: (a) $Pd_2(dba)_3$, QPhos, Et_3N, DMF, 90 °C, 18 h, **3a**: 33%, **3b**: 33%.

The prepared compounds were found to be stable for months without significant formation of degradation products when stored at a dark place at −20 °C under argon atmosphere. Stability tests by UV/Vis spectroscopy in water containing 1% DMSO showed that the dyes are also stable in solution for at least three hours (Figures S45 and S46). We observed a significant quenching effect of our tetrazine-modified dyes in comparison to their published unmodified counterparts (Table S1): The quantum yields of **3a** and **3b** are reduced to 0.4% and 0.3%, respectively.

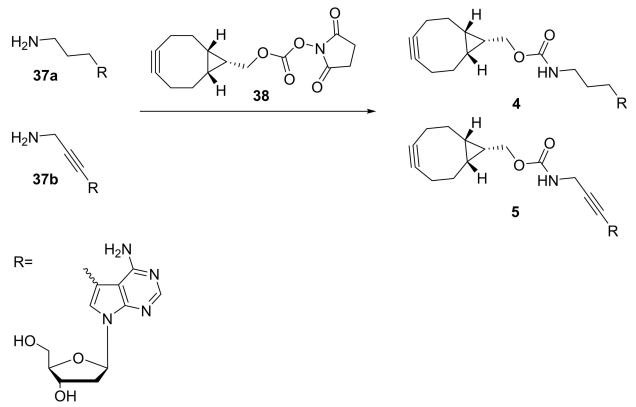
To investigate the potential of tetrazine-modified cyanine-styryl dyes, we synthesized a selection of four different BCN modified nucleosides **4**–**7** based on uridine or 7-deaza-2′-deoxyadenosine, respectively (Schemes 2, 4 and 6, Figures S72–S99). The nucleoside precursors (Scheme 4: **37a/b**, **47** and **48**) contain terminal amine functions at their respective linkers and were prepared by known procedures and coupled with the commercially available BCN-NHS ester **38**.[27–29] We chose this set of nucleosides to investigate the effects of two different linkages (flexible propyl vs. rigid propynyl) and the influence of two different nucleosides (uridine vs. 7-deaza-2′-deoxyadenosine) on the turn-on rates and on the kinetics of the iEDDA reaction with the dyes **3a** and **3b**. We wanted to study our



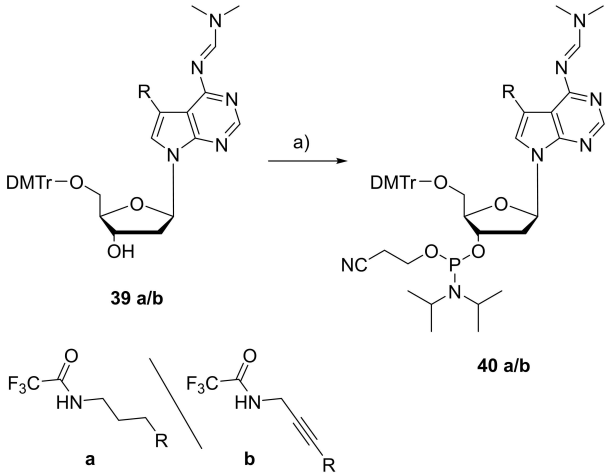
Scheme 2. BCN-modified nucleosides **4**–**7** and **DNA2**, **DNA4** and **DNA6**.



Scheme 3. Synthesis of tetrazine-modified cyanine-styryl dyes **3a** and **3b**: a) $POCl_3$, DMF, 0 °C, 2 h, 93%. b) K_2CO_3, $(CH_3)_2CO_3$, DMF, 130 °C, 19 h, 97%. c) Piperidine, EtOH, 80 °C, 18 h, **2a**: 88%, **2b**: 98%. d) ZnI_2, $H_4N_2 \cdot H_2O$, 60 °C, 23 h, 48%. e) Methanesulfonyl chloride, Et_3N, CH_2Cl_2, r.t., 30 min, 91%. f) $Pd_2(dba)_3$, QPhos, Et_3N, DMF, 100 °C, 18 h, **3a**: 33%, **3b**: 33%.



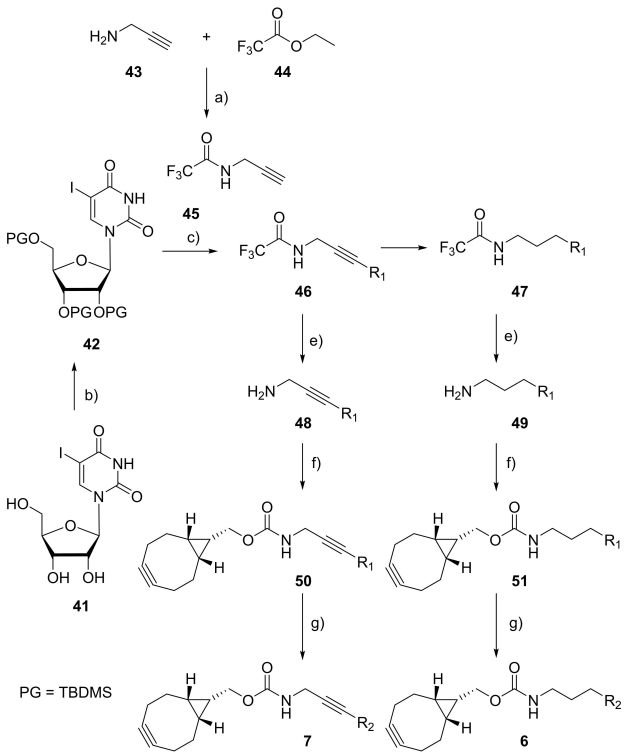
Scheme 4. Synthesis of the BCN-modified 2′-deoxyadenosines **4** and **5**: Et_3N, **38**, DMF_{dry}, RT, 16 h, **4**: 60%, **5**: 70%.



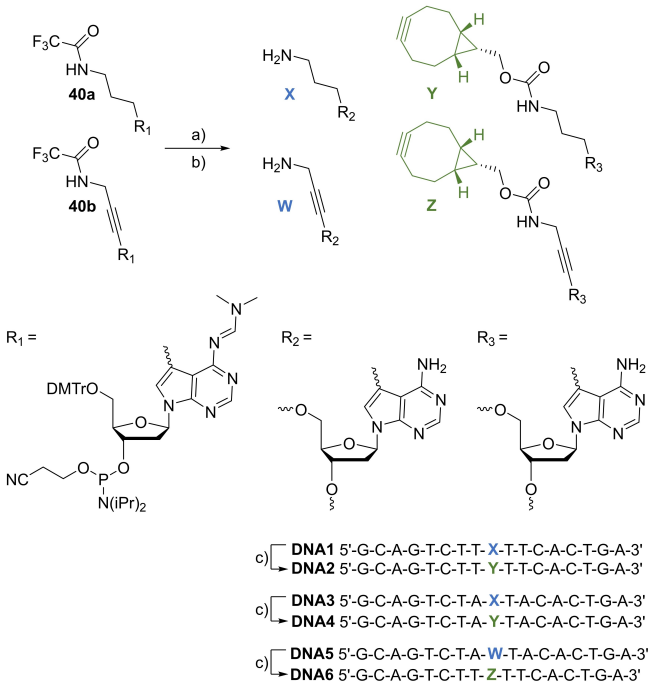
Scheme 5. Synthesis of the phosphoramidites **40a** and **40b**. a) $(iPr)_2NEt$, 2-cyanoethyl-*N*,N-diisopropylchlorophosphor-amidite, 3.5 h, r.t., quant.

iEDDA labelling also on an oligonucleotide and employed the phosphoramidites **40a** and **40b** (Scheme 5) that we synthesized according to literature and incorporated them into the oligonucleotides by standard solid-phase and automated DNA synthesis.[28] The precursors **DNA1**, **DNA3** and **DNA5** were postsynthetically modified with the BCN-NHS ester **38**, leading to the BCN-modified **DNA2**, **DNA4** and **DNA6** (Scheme 7, Figures S1–S6).

The "click" products **8**–**29** formed by the iEDDA reaction (Table 1) were confirmed by ESI mass spectrometry (Table S2 and Figures S100–S111). We chose the iEDDA reaction with unsubstituted BCN-OH as the reference for evaluating the basic fluorogenicity and reactivity of our synthesized dyes **3a** and **3b**. The influence of the nucleobases **4**–**7** compared to unsubstituted BCN-OH on the reactivity was remarkably different. While the BCN-modified uridine **6** shows only little increases of the second-order rate constant between two- to three-fold, the BCN-modified 7-deaza-2′-deoxyadenosines **4** and **5** had a much higher impact and showed a six-fold increase of the reaction rate with both tetrazine-modified dyes (Figure 2). A template



Scheme 6. Synthesis of the BCN-modified 2′-deoxyuridines **6** an **7**: a) MeOH, 0 °C, 30 Minuten, r.t., 18 h, 68 %; b) TBDMS–Cl, imidazole, pyridine, 50 °C, 2 h, 92 %; c) $Pd(PPh_3)_4$, CuI, Et_3N, THF, 50 °C, 3 h, 80 %; e) NaOH, MeOH, r.t., 2 h, **48**: 70 %, **49**: %; f) **38**, Et_3N, THF, r.t., 19 h, **50**: 73 %, **51**: 57 %; g) Et_3N-HF, THF, rt, 18 h, **7**: 76 %, **6**: 51 %. R_1 = TBDMS-protected uridine, R_2 = uridine.



Scheme 7. Synthesis of **DNA1**, **DNA3**, **DNA5**, and the BCN-modified **DNA2**, **DNA4**, **DNA6** starting from phosphoramidite **40a** and **40b**, respectively. (a) DNA synthesizer, (b) 25 % aq. NH_4OH, 18 h, 55 °C, (c) **25**, DMSO, Et_3N, r.t., 18 h.

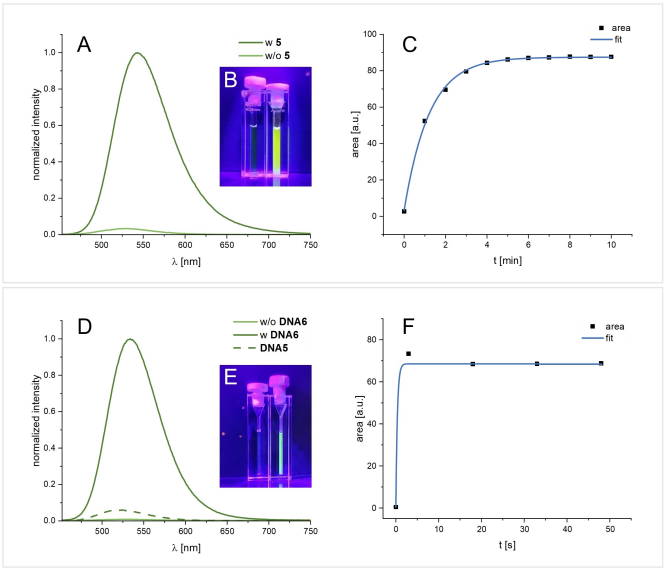
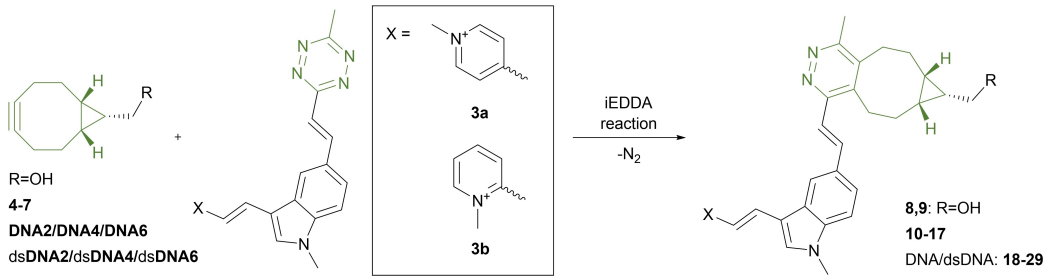


Figure 2. A) Increase of fluorescence intensity (λ_{exc} = 437 nm) during the reaction of nucleoside **5** (100 µM, 5.0 equiv.) with dye **3a** (20 µM) in H_2O (with 1 % DMSO). B) Visible turn-on effect: **3a** before (left) an after the reaction with **5** (right). C) Kinetic plot with exponential fit function $y = a + b\exp(-kx)$. D) Increase of fluorescence intensity (λ_{exc} = 437 nm) during the reaction of **DNA6** (10 µM) with dye **3a** (2 µM, 5.0 equiv.) in H_2O (with 1 % DMSO). E) Visible turn-on effect: **3a** before (left) and after the reaction with **DNA6** (right). F) Kinetic plot with exponential fit function $y = a + b\exp(-kx)$. For the kinetics of the other reactions, see Figures S11–S32.

effect of the 7-deaza-2′-deoxyadenosine moiety in **4** and **5** might favor the iEDDA reaction. We assume that an influence of the missing 2′-hydroxyl group at the 7-deaza-2′-deoxyadenosine **4** and **5** can be excluded, due to the large spatial separation of the reactive center from the 2′-position. With **DNA2**, to our surprise, we observed an immense increase in second-order rate constants by up to a 1000-fold compared to the nucleoside **4**. The kinetics do not depend on the sequence because the **DNA4** with a slightly altered sequence around the BCN-modification site is labelled comparably fast (Figure 3).

Additionally, by introducing the sterically more demanding nucleosides to the BCN moiety in **4**–**7**, we noticed an increase of the turn-on factors of the dyes in comparison to the unsubstituted BCN-OH by a factor of approximately two. It is worth noticing that, while the tetrazine-modified cyanine-styryl dyes **3a** and **3b** show a rather similar behaviour with a turn-on effect of 12 in the corresponding iEDDA reaction with BCN-OH, the tetrazine-modified dye **3a** shows generally higher quantum yields across all conducted experiments. This leads us to the conclusion, that dye **2a** is more suitable for the cell imaging applications, due to its higher brightness of $2.0 \times 10^3\ L\,mol^{-1}\,cm^{-1}$ (Table S1). After comparing the results of the BCN-modified nucleosides **4**–**7**, we concluded that the 7-deaza-2′-deoxyadenosine-derivative **4** is suited for further experiments, since it reacts very selectively with a second-order rate constant of $43\ M^{-1}s^{-1}$ and $30\ M^{-1}s^{-1}$, respectively. Moreover, the combination of the rigid propynyl linker and the 7-deaza-2′-deoxyadenosine in the nucleoside **5** yields the optimal turn-on fluorescence of 32 and 54 with the dyes **3a** and **3b**,

Table 1. Photophysical properties of the dyes **3a** and **3b** after their complete conversion with the BCN-OH, the BCN-modified substrates **4–7**, **DNA2**, **DNA4** and **DNA6** in H_2O/DMSO (99:1 V/V%), **dsDNA2**, **dsDNA4** and **dsDNA6** in H_2O/DMSO (99:1 V/V%, 250 mM NaCl, 10 mM K-P_i buffer).



Dye	BCN-R	Product	λ_{em} [nm]	$\Delta\lambda$ [eV][c]	Φ_F[d]	Turn-on	k_2 [M^{-1} s^{-1}]
3a[a]	R=OH	8	539	0.537	0.073	12	7.0
	4	10	541	0.545	0.068	19	43
	5	12	**543**	0.554	0.072	32	137
	6	14	539	0.537	0.071	27	9.6
	7	16	542	0.549	0.071	27	15
	DNA2	18	**528**	**0.489**	**0.121**	**58**	≥ 125,000
	dsDNA2[e]	20	**545**	**0.562**	**0.272**[f]	**340**	≥ 69,900
	DNA4	22	**534**	**0.515**	**0.142**	**75**	≥ 113,000
	dsDNA4[e]	24	**546**	**0.566**	**0.232**[f]	**270**	≥ 159,000
	DNA6	26	**534**	**0.515**	**0.164**	**170**	≥ 284,000
	dsDNA6[e]	28	**544**	**0.558**	**0.274**[f]	**290**	≥ 106,000
3b[b]	R=OH	9	523	0.553	0.022	12	5.0
	4	11	529	0.580	0.018	22	30
	5	13	**537**	**0.615**	**0.023**	**54**	**83**
	6	15	529	0.580	0.022	16	6.6
	7	17	533	0.598	0.023	42	12
	DNA2	19	513	0.507	0.027	49	≥ 110,000
	ds**DNA2**[e]	21	527	0.571	0.154[f]	490	≥ 105,000
	DNA4	23	515	0.517	0.030	50	≥ 134,000
	dsDNA4[e]	25	524	0.558	0.110[f]	310	≥ 186,000
	DNA6	27	519	0.535	0.049	130	≥ 221,000
	dsDNA6[e]	29	526	0.567	0.196[f]	560	≥ 110,000

[a] λ_{exc} = 437 nm, ε_{449nm} = 26,400 L mol^{-1} cm^{-1} (Figure S9). [b] λ_{exc} = 424 nm, ε_{436nm} = 23,200 L mol^{-1} cm^{-1} (Figure S10). [c] Stokes shift. [d] If not stated otherwise, quantum yields were measured in DMSO. [e] ds = double stranded; annealed with 1.1 equiv. of unmodified counterstrand at 90 °C for 10 min, slow cooling to r.t. [f] Quantum yields of the dsDNA were measured in a mixture of H_2O with 1% DMSO, 60 mM NaCl and 2.5 mM of a K-P_i buffer.

respectively. The turn-on with **DNA2** also increased by an additional factor of two to three compared to the corresponding nucleoside **4**. In fact, we were not able to experimentally follow in detail the slope in the course of the iEDDA reaction since it already showed complete conversion after 3 seconds. The fluorescence turn-on with **DNA4** is similar and thus does not depend on the sequence.

To explain our observation, we assumed a DNA-templated pre-coordination of the dyes **3a** and **3b** due to the electrostatic attraction of the positively charged dyes and the negatively charged DNA. To verify this hypothesis, we monitored the unmodified **DNA1** and **DNA5** with each of the unmodified cyanine-styryl dyes **2a** and **2b** (Figures S33, S34 and S37, S38) and tracked the change of the fluorescence intensity. We hypothesized that the non-covalent pre-coordination of the positively charged dyes with the negatively charged DNA precedes the iEDDA reactions and the additional spatial constraint imposed by the DNA would lead to a slight increase in turn-on rates due to the restriction of the non-fluorescent depopulation of the excited state. We were pleased to confirm this template effect as plausible, since all tested dyes showed a fluorescence turn-on within 2 minutes. The fluorescence turn-on is further increased to values between 270 and 490 when the double-stranded (ds) BCN-modified ds**DNA2** and ds**DNA4** were labelled with **3a** and **3b**. This further supports our concept of the two-factor fluorogenicity: Not only the reaction of the tetrazine releases fluorescence, the interactions of the cyanine-styryl dyes with the DNA enhance the fluorescence intensity, too.

Finally, we incorporated the BCN group into **DNA6** and ds**DNA6** by means of a slightly different linker. The linker contained the more rigid propargyl linker instead of the flexible propynyl linker in **DNA2** and **DNA4**. It shows even faster reactions (up to $k_2 \geq 284{,}000$ M^{-1} s^{-1}) and higher fluorescence turn-on ratios (up to 560). Taken together, this shows that the way how the BCN is attached as reactive group to the DNA is more important than sequence variations. The very fast kinetics and the high fluorescence turn-on for the reaction of the BCN-labelled DNA with the dyes **3a** and **3b** is the prerequisite for the subsequent application of in vivo labelling of DNA and live cell imaging.

To proof the biocompatibility of the described iEDDA reaction HeLa cells were transfected for 24 h with 75 nM of **DNA2** using Lipofectamine® as transfection reagent. After subsequent treatment of the HeLa cells with 150 nM of either the dyes **3a** (Figure 4) or **3b** (Figure S47) for 60 min, we imaged

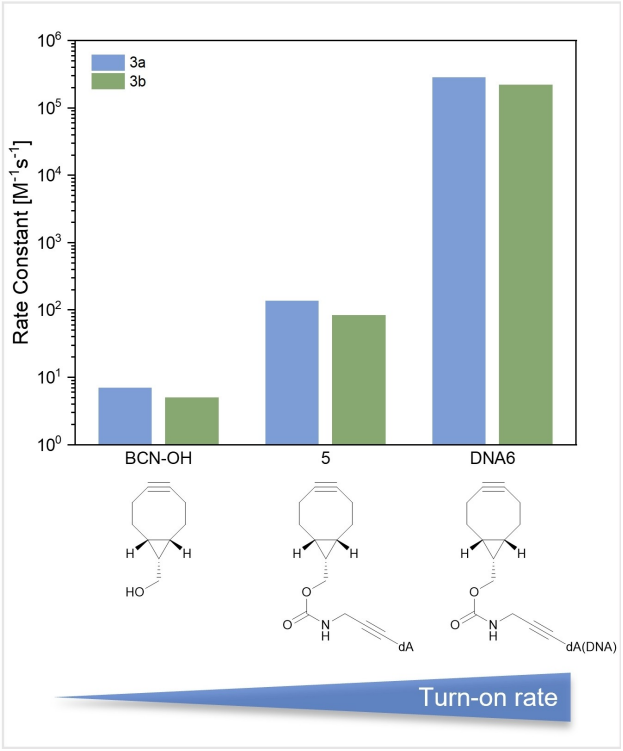


Figure 3. Comparison of the second-order rate constants of the iEDDA reactions between tetrazine-modified cyanine-styryl **3 a**, **3 b** and three BCN-conjugates BCN-OH, **5**, **DNA6**.

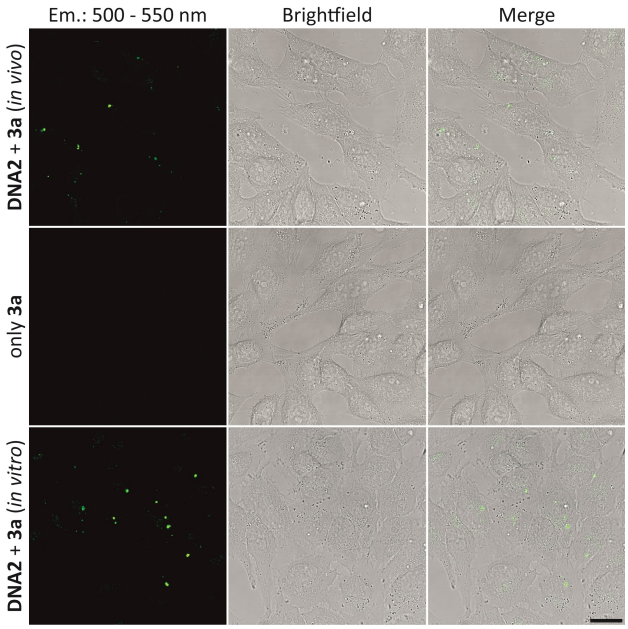


Figure 4. HeLa cells after 24 h transfection with **DNA2** (15 pmol) and subsequent labelling with **3 a** for 1 h (top). Cells only treated with **3 a** served as a negative control (middle), whereas cells transfected with **DNA2 + 3 a** (in vitro reaction before transfection) represent the positive control (bottom). Imaging by confocal fluorescence microscopy with a 488 nm laser line and an emission channel at 500–550 nm, complemented with a brightfield channel. Scale bar: 30 μm.

the cells via confocal fluorescence microscopy. Without an additional washing step, the "click"-product could be visualized in both the endosomes and also in the cytosol (after endosomal escape) in the living cells.

As a negative control, we treated the HeLa cells only with 150 nM of dye **3 a** but did not transfect them before with **DNA2**. By this experiment, we can exclude the fluorescence signal of unbound **3 a** from the "click"-product. Furthermore, we conducted a positive control experiment by performing the iEDDA reaction with the BCN-modified nucleic acid **DNA2** and dye **3 a** in vitro before transfecting the resulting DNA conjugate into HeLa cells and determined the observed fluorescence as a successful transfected "click"-product. We were pleased to see a similar fluorescence pattern in this control experiment as previously for the in vivo labelling experiment. This result highlights the significant fluorescence gain by the selective and efficient and labelling of the BCN-modified **DNA2** with the tetrazine-modified dyes **3 a** (Figure 4) and **3 b** (Figure S47), while reducing the signal-to-noise ratio. This opens interesting possibilities for the application of tetrazine-modified dyes in live cell imaging and future metabolic labelling experiments.

Conclusion

In conclusion our synthetic tetrazine-modified cyanine-styryl dyes **3 a** and **3 b** are stable in the solid state over months and in aqueous solution as well as cell lysate for hours. As basic features, the cyanine-styryl dyes are highly photostable[21,22] and show high Stokes shifts of up to 0.60 eV separating excitation and emission very well. The dyes undergo iEDDA reactions with the BCN-modified nucleosides **4**–**7** at moderate to good reaction rate constants of $k_2 = 6.6$ to 137 $M^{-1}s^{-1}$. These reactions are significantly accelerated with DNA bearing a BCN-nucleotide unit and show very high rate constants of $\geq$284,000 $M^{-1}s^{-1}$. There is a clear correlation between the size of the BCN-reaction partner and the iEDDA reaction. Additionally, the fluorescence turn-on increases from 12 to 130, which is of great potential for live cell imaging and metabolic labeling applications. By hybridizing the DNA single strands with their corresponding counterpart strands, we were able to further increase the fluorescence turn-on up to 560-fold. This fluorogenicity is the result of our concept of the two-factor fluorescence release. Assumingly, the dyes pre-coordinate in a templated fashion, which enhances the second-order rate constant k_2 by a factor of 1000 and enhances the fluorescence by a factor of 2 to 5. Experiments in living HeLa cells have shown that the dyes **3 a** and **3 b** are cell permeable even in low concentrations and are able to undergo iEDDA reactions with the BCN-modified **DNA2** in the intracellular environment. The fluorogenicity concept of these dyes allows imaging of DNA in living cells with such an improved signal-to-noise ratio that washing procedures are not required prior to cell imaging, which is important for cell imaging microscopy. This is a great advantage for live cell imaging of DNA and RNA, for example by means of metabolic labelling, in the future.

Experimental Section

Materials: All starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Alfa Aesar) and used without further purification. All reactions were carried out under a dry argon environment. All solvents were of reagent grade and used as received. Chemicals and reagents were used as commercially supplied without any further purification unless otherwise stated. Room temperature refers to ambient temperature (20–22 °C). Reactions were monitored by Thin Layer Chromatography (TLC) using aluminum backed silica gel 60 (F254) plates, visualized using UV254 nm and 365 nm and potassium permanganate dips as appropriate. Column chromatography was carried out using silica gel G60 (Fluka analytical, 230–400 mesh, 40–63 μm particle size, 60 Å) as the stationary phase. HeLa cells were bought ATCC (Manassas Virginia).

NMR spectroscopy: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer and a Bruker 500 MHz spectrometer. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. Coupling constants (*J*) are reported in Hertz (Hz) without adjustments; therefore, due to limits in digital resolution, in some cases there are small differences ($<$ 1 Hz) in the measured J value of the same coupling constant determined from different signals. Splitting patterns are designed as follows: s – singlet, d – doublet, t – triplet, dd – doublet of doublets, dt – doublet of triplets, td – triplet of doublets, ddd – doublet of doublet of doublets, tt – triplet of triplets, sp – septet, hept – heptet, m – multiplet, br – broad. Various 2D techniques experiments were used to establish the structures and to assign the signals.

Mass spectrometry: High-resolution mass spectra were obtained with an electrospray ionization Thermo Exactive orbitrap mass spectrometer. Some of mass spectra were also measured on a Shimadzu Axima Confidence using 3-hydroxypicolinic acid as matrix substance.

Optical spectroscopy: Spectroscopic measurements were carried out on a Cary 100 Scan UV/vis spectrometer by Varian and a Fluoromax-4 spectrofluorometer by Horiba Jobin-Yvon.

Oligonucleotide synthesis: Oligonucleotide synthesis was performed on a H-6 synthesizer by K&A Laborgeräte. After cleavage, the oligonucleotides were purified by a standard Glen Gel-Pak™ desalting protocol. The purified DNA was used for the post-synthetic BCN coupling and purified on a semi-preparative reversed-phase HPLC ThermoFisher system (RP–C18 column, A = NH_4HCO_3 buffer, B = acetonitrile). The purified oligonucleotide strands were quantified photometrically using a NanoDrop ND-1000 spectrometer.

Synthesis of the tetrazine-modified cyanine-styryl dyes 3 a and 3 b

31: Under argon, 5-bromoindole (**30**) (4.50 g, 23.0 mmol, 1.00 equiv.) and $POCl_3$ (9.50 g, 62.0 mmol, 2.70 equiv.) were dissolved in 2.5 mL dry DMF each. The 5-bromoindole solution was added to the $POCl_3$ solution at 0 °C and stirred for 2 h. Then the reaction mixture was added to 40 mL of ice water. A neutral to slightly basic pH was adjusted with 2 M NaOH (up to pH 9). The solution was extracted three times with EtOAc and the aqueous phase was washed with saturated NaCl solution. The organic phase was dried with Na_2SO_4 and the solvent removed under reduced pressure to obtain an off-white solid (4.80 g, 93 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.30 (s, 1H), 9.93 (s, 1H), 8.33 (s, 1H), 8.23 (s, 1H), 7.49 (d, *J* = 8.6 Hz, 1H), 7.38 (dd, *J* = 8.6, 2.0 Hz, 1H).^{13}C NMR (101 MHz, DMSO-d6): δ = 185.1, 139.2, 135.8, 126.1, 125.9, 123.0, 117.5, 114.9, 114.6. HR-ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_9H_7BrNO^+$: 223.97055; found: 223.97095.

32: **31** (4.80 g, 21.4 mmol, 1.00 equiv.) and K_2CO_3 (3.26 g, 23.6 mmol, 1.10 equiv.) were suspended in dry DMF to which dimethyl carbonate (7.21 mL, 7.72 g, 85.7 mmol, 4.00 equiv.) was added. The suspension was stirred at 130 °C for 19 h. After cooling to r.t., the mixture was poured on 50 mL of ice water and the aqueous solution was extracted three times with EtOAc. The organic layer was washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure to yield a beige-colored solid (4.96 g, 97 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.87 (s, 1H), 8.27 (s, 1H), 8.21 (d, *J* = 1.9 Hz, 1H), 7.52 (d, *J* = 8.7 Hz, 1H), 7.43 (dd, *J* = 8.7, 2.0 Hz, 1H), 3.86 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6): δ = 184.5, 142.2, 136.4, 126.2, 126.0, 123.0, 116.2, 115.3, 113.1, 33.5. HR-ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{10}H_9BrNO^+$: 237.98320; found: 237.98659.

2 a: 1,4-dimethylpyridinium iodide (**33 a**) (1.00 g, 4.25 mmol, 1.00 equiv.) was placed in a round bottom flask under an argon atmosphere and suspended in 40 mL ethanol (EtOH). The suspension was stirred for 10 min. **32** (1.52 g, 6.38 mmol, 1.50 equiv.) and piperidine (2.10 mL, 21.3 mmol, 5.00 equiv.) were added to the suspension and stirred overnight at 80 °C. After the reaction mixture had cooled to r.t., Et_2O was added until no more solid precipitated. The precipitate was washed three times with diethyl ether and dried in vacuo to obtain an orange powder (1.70 g, 88 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.69 (d, *J* = 6.8 Hz, 2H), 8.32 (d, *J* = 1.7 Hz, 1H), 8.17 (d, *J* = 16.3 Hz, 1H), 8.12 (d, *J* = 6.9 Hz, 2H), 8.01 (s, 1H), 7.52 (d, *J* = 8.7 Hz, 1H), 7.40 (td, *J* = 8.7, 1.8 Hz, 1H, 5), 7.20 (d, *J* = 16.3 Hz, 1H), 4.19 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 153.8, 144.1, 136.5, 135.5, 134.4, 127.2, 125.3, 122.4, 121.8, 117.4, 114.3, 112.9, 112.0, 46.3, 33.3. HR-ESI-MS (*m/z*): $[M]^+$ calcd. for $C_{17}H_{16}BrN_2^+$: 327.04914; found: 327.04909.

2 b: 1,2-dimethylpyridinium iodide (**33 b**) (1.00 g, 4.25 mmol, 1.00 equiv.) was placed in a round bottom flask under an argon atmosphere and suspended in 40 mL EtOH. The suspension was stirred for 10 min. Then **32** (1.52 g, 6.38 mmol, 1.50 equiv.) and piperidine (2.10 mL, 21.3 mmol, 5.00 equiv.) were added to the suspension and stirred overnight at 80 °C. After the reaction mixture had cooled to r.t., Et_2O was added until no more solid precipitated. The precipitate was washed three times with Et_2O and dried in vacuo to obtain an orange powder (1.91 g, 98 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.75 (d, *J* = 5.8 Hz, 1H), 8.54 (d, *J* = 7.8 Hz, 1H), 8.31 (d, *J* = 11.6 Hz, 2H), 8.28 (d, *J* = 1.7 Hz, 1H), 8.22 (d, *J* = 15.8 Hz, 1H), 7.72–7.65 (m, 1H), 7.52 (d, *J* = 8.7 Hz, 1H), 7.39 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.20 (d, *J* = 15.8 Hz, 1H), 4.30 (s, 3H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 153.3, 145.0, 142.8, 136.2, 136.2, 135.2, 127.6, 125.3, 123.4, 122.8, 122.0, 114.3, 112.9, 111.9, 110.9, 56.0, 45.7, 33.5, 18.5. HR-ESI-MS (*m/z*): $[M]^+$ calcd. for $C_{17}H_{16}BrN_2^+$: 327.04914; found: 327.04847.

36: 3-hydroxypropionitrile (**34**) (0.190 mL, 2.81 mmol, 1.00 equiv.), zinc iodide (0.269 g, 0.844 mmol, 0.30 equiv.) and MeCN (**35**) (0.730 mL, 14.1 mmol, 5.00 equiv.) were suspended in 5.00 mL 1,4-dioxane under an argon atmosphere. Hydrazine monohydrate (6.97 mL, 141 mmol, 50.0 equiv.) was added to this suspension, and the reaction mixture was stirred at 60 °C. for 23 h. After the solution had cooled down, $NaNO_2$ (0.500 g, 7.25 mmol, 2.57 equiv.) was first dissolved in 10 mL of ice-cold H_2O and added to the reaction solution. 1 M HCl was added until gas evolution was no longer observed (pH = 3). The pink solution was extracted three times with 300 mL EtOAc and the combined organic layers were dried over Na_2SO_4. The solvent was removed under reduced pressure and purified via column chromatography on silica gel (hexane/EtOAc 1 : 1). The product was obtained as a dark red oil (0.190 g, 48 %). ^{1}H NMR (400 MHz, chloroform-d): δ = 4.25 (t, *J* = 5.7 Hz, 2H), 3.56 (t,

J = 5.8 Hz, 2H), 3.05 (s, 3H), 2.50 (s, 1H). ^{13}C NMR (101 MHz, chloroform-d): δ = 168.4, 168.0, 60.1, 37.5, 21.3. HR-ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_5H_9N_4O^+$: 141.07709; found: 141.07707.

1: **36** (0.461 g, 3.29 mmol, 1.00 equiv.) and dry Et_3N (0.64 mL, 4.61 mmol, 1.40 equiv.) were dissolved in 15.0 mL dry CH_2Cl_2 under an argon atmosphere. Then methanesulfonyl chloride (0.36 mL, 4.61 mmol, 1.40 equiv.) was added and the mixture was stirred at r.t. for 30 min. The progress of the reaction was checked by means of TLC. After the reaction was completed, the solution was diluted with another 15.0 mL of CH_2Cl_2 and washed twice with water. The organic layer was dried over Na_2SO_4. After the solvent has been removed under reduced pressure, the crude product was purified via column chromatography (hexane/EtOAc 1:1) to obtain a pink solid (0.654 g, 91%). ^{1}H NMR (400 MHz, chloroform-d): δ = 4.87 (t, J = 6.2 Hz, 2H), 3.76 (t, J = 6.2 Hz, 2H), 3.08 (s, 3H), 3.02 (s, 3H). ^{13}C NMR (101 MHz, chloroform-d): δ = 168.3, 166.2, 66.1, 37.7, 34.7, 21.3. HR-ESI-MS (*m/z*): [M–H]- calcd. for $C_6H_9N_4O_3S^+$: 217.04008; found: 217.03965.

3a: **2a** (0.106 g, 0.232 mmol, 1.00 equiv.), $Pd_2(dba)_3$ (0.012 g, 0.012 mmol, 10 mol%) and QPhos (0.034 g, 0.048 mmol, 40 mol%) were added to a 10 mL crimp-vial and dried under high vacuum. The solids were then solved with 1.00 mL dry DMF. Subsequently, 1.00 mL of a 0.24 M tetrazine stock solution (**1**). and dry Et_3N (0.16 mL, 1.16 mmol, 5.00 equiv.) were added to the solution. The reaction took place in a metal heating block at 80 °C for 18 h. After the solution has cooled to r.t., the solution was mixed with an excess of an aqueous solution of KPF_6 and stirred vigorously for 15 min. The organic solvent was removed under reduced pressure. The aqueous solution was then diluted with CH_2Cl_2 and washed three times with water. The organic phase was dried over Na_2SO_4. After the solvent has been removed, the crude product was purified via column chromatography (CH_2Cl_2/MeOH 95:5) to obtain an orange solid (0.04 g, 33%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.72 (d, J = 6.7 Hz, 2H), 8.62 (s, 1H), 8.47 (d, J = 16.3 Hz, 1H), 8.23 (d, J = 16.3 Hz, 1H), 8.17 (d, J = 6.8 Hz, 2H), 8.00 (s, 1H), 7.94 (dd, J = 8.7, 1.5 Hz, 1H), 7.71 (t, J = 16.3 Hz, 1H), 7.68 (d, J = 8.6 Hz, 1H), 7.41 (t, J = 16.3 Hz, 1H), 4.19 (s, 3H), 3.92 (s, 3H), 2.96 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ = 165.9, 164.5, 154.0, 144.3, 141.1, 138.9, 136.3, 134.9, 128.7, 125.7, 122.1, 121.9, 118.7, 117.7, 113.3, 111.8, 46.3, 33.3, 20.8. HR-ESI-MS (m/z): $[M]^+$ calcd. for $C_{22}H_{21}N_6^+$: 369.18222; found: 369.18169.

3b: **2b** (0.106 g, 0.232 mmol.1.00 equiv.), $Pd_2(dba)_3$ (0.012 g, 0.012 mmol, 10 mol%) and QPhos (0.034 g, 0.048 mmol, 40 mol%) were added to a 10 mL crimp-vial and dried under high vacuum. The solids were then solved with 1.00 mL dry DMF. Subsequently, 1.00 mL of a 0.24 M tetrazine stock solution (**1**). and dry Et_3N (0.16 mL, 1.16 mmol, 5.00 equiv.) were added to the solution. The reaction took place in a metal heating block at 80 °C for 18 h. After the solution has cooled to r.t., the solution was mixed with an excess of an aqueous solution of KPF_6 and stirred vigorously for 15 min. Thereafter, the organic solvent was removed under reduced pressure. The aqueous solution was then diluted with CH_2Cl_2 and washed three times with water. The organic phase was dried over Na_2SO_4. After the solvent has been removed, the crude product was purified via column chromatography (CH_2Cl_2/MeOH 95:5) to obtain an orange solid (0.04 g, 33%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.79 (d, J = 6.2 Hz, 1H), 8.59 (s, 1H), 8.54 (d, J = 8.6 Hz, 1H), 8.47 (d, J = 16.3 Hz, 1H), 8.40 (t, J = 7.9 Hz, 1H), 8.28 (d, J = 15.8 Hz, 1H), 8.20 (s, 1H), 7.94 (d, J = 8.6 Hz, 1H), 7.74 (t, J = 6.8 Hz, 1H), 7.71 (d, J = 16.5 Hz, 1H), 7.68 (d, J = 7.8 Hz, 1H), 7.36 (dd, J = 90.0, 15.8 Hz, 1H), 4.38 (s, 3H), 3.95 (s, 3H), 2.96 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ = 165.9, 164.5, 153.6, 145.3, 143.1, 141.1, 138.7, 136.7, 136.1, 128.7, 126.0, 123.4, 123.0, 122.7, 121.5, 118.8, 113.2, 111.8, 111.2, 54.9, 45.7, 33.4, 20.8. HR-ESI-MS (m/z): $[M]^+$ calcd. for $C_{22}H_{21}N_6^+$: 369.18222; found: 369.18163.

Synthesis of BCN-modified 2′-deoxyadenosines 4 and 5

4 and 5: **37a/b** (0.037 g, 0.121 mmol, 1.00 equiv.) was dissolved in 6.00 mL of drxy DMF. Triethylamine (0.067 mL, 0.484 mmol, 4.00 equiv.) and BCN-NHS ester **38** (0.053 g, 0.182 mmol, 1.50 equiv.) were added and the reaction solution was stirred for 16 h at r.t. The solvent was removed under reduced pressure. The residue was dissolved in 50.0 mL MeOH, Amberlite IRA 402 bicarbonate was added and stirred for 30 min. The solution was filtered off and the solvent was removed under reduced pressure. The product **4** was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH 9:1). A light-yellow powder (0.035 g, 60%) was obtained. ^{1}H NMR (400 MHz, methanol-d_4): δ = 8.11 (s, 1H), 8.03 (s, 1H), 7.14 (s, 1H), 6.52–6.42 (m, 1H), 4.51 (dt, J = 5.3, 2.3 Hz, 1H), 4.33–4.27 (m, 1H), 4.15 (d, J = 8.1 Hz, 2H), 3.99 (q, J = 3.3 Hz, 1H), 3.82–3.68 (m, 2H), 3.19 (t, J = 6.8 Hz, 2H), 2.81 (t, J = 7.4 Hz, 2H), 2.70–2.62 (m, 3H), 2.29 (dd, J = 5.7, 2.4 Hz, 1H), 2.24 (s, 1H), 2.21 (s, 1H), 2.18 (s, 1H), 2.14 (s, 1H), 1.86 (dt, J = 13.0, 6.0 Hz, 2H), 1.61 (d, J = 12.8 Hz, 1H), 1.52–1.46 (m, 1H), 1.37 (d, J = 8.3 Hz, 2H), 1.29 (s, 1H), 1.00–0.92 (m, 3H). ^{13}C NMR (101 MHz, methanol-d_4): δ = 159.2, 151.9, 151.2, 130.6, 121.4, 116.5, 104.4, 99.5, 88.9, 86.3, 73.1, 68.7, 63.8, 63.7, 41.2, 41.0, 40.4, 40.3, 31.7, 30.2, 21.9, 21.4, 19.0, 14.4, 11.4. HR-ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{25}H_{33}N_5O_5^+$: 484.25544; found: 484.25511. The product **5** was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH 9:1). A light-yellow powder (0.041 g, 70%) was obtained. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.10 (s, 1H), 7.71 (d, J = 10.5 Hz, 2H), 6.50–6.43 (m, 1H), 5.35–5.21 (m, 1H), 5.08 (t, J = 5.0 Hz, 1H), 4.34 (s, 1H), 4.15–4.08 (m, 3H), 4.02 (d, J = 5.3 Hz, 2H), 3.87–3.78 (m, 1H), 3.54 (dtd, J = 20.8, 11.4, 4.4 Hz, 2H), 3.17 (d, J = 4.9 Hz, 3H), 2.47–2.41 (m, 1H), 2.23–2.11 (m, 5H), 1.55 (d, J = 9.8 Hz, 2H), 1.29 (p, J = 8.3 Hz, 1H), 0.86 (t, J = 9.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 157.5, 156.6, 152.7, 149.2, 125.9, 102.3, 99.0, 94.6, 89.3, 87.5, 83.2, 75.3, 71.0, 62.0, 61.9, 48.6, 39.9, 30.9, 28.6, 20.9, 19.6, 17.6. HR-ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{25}H_{30}N_5O_5^+$: 480.22415; found: 480.22412.

Synthesis of the phosphoramidites 40a and 40b

39a/b (0.100 g, 131 μmol, 1.00 equiv.) was dissolved in 5.00 mL dry CH_2Cl_2 to which 67.1 μL (iPr)$_2$NEt (67.1 μL, 394 μmol, 3.00 equiv.) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (58.6 μL, 263 μmol, 2.00 equiv.) were added and stirred for 3.5 h at r.t. The reaction solution was then directly purified by column chromatography (SiO_2, CH_2Cl_2/acetone 1:1 + 0.1% Et_3N) to obtain a colorless foam (120 mg, 131 μmol, quant.). ^{31}P NMR (162 MHz, DMSO-d_6): δ = 147.54, 147.00, 13.89. The spectroscopic data are in agreement with the literature.[30]

Synthesis of the BCN-modified 2′-deoxyuridines 6 and 7

45: To a solution of 2.00 mL propargylamine (**43**) (1.72 g, 31.2 mmol, 1.00 equiv.) in 30 mL absolute MeOH at 0 °C was slowly added 4.46 mL ethyl trifluoroacetate (**44**) (5.31 g, 37.4 mmol, 1.20 equiv.). After 30 min, the ice bath was removed, and the mixture was stirred at r.t. for 18 h. The solvent was removed under reduced pressure and the residue was dissolved in 50 mL of CH_2Cl_2. The solution was then washed twice with saturated $NaHCO_3$ solution (50 mL). The aqueous phase was extracted twice with 30 mL of CH_2Cl_2. The combined organic phases were washed with 50 mL of water and dried over $MgSO_4$. The solvent was removed under reduced pressure. The crude product was purified via column chromatography o silica gel (cyclohexane/EtOAc 5:1). The product was obtained as a colorless oily liquid (2.53 g, 68%). ^{1}H NMR (400 MHz, chloroform-d): δ (ppm) = 6.53 (br. s, 1H), 4.16 (dd, J = 5.4, 2.6 Hz, 2H), 2.34 (m, 1H). HR-MS (ESI): calcd. for $C_5H_4F_3NO$ $[M]^+$:

151.02450; found $[M+H]^+$: 152.03160. The spectroscopic data agree with the literature.[31]

42: 5-ioduridine (**41**) (1.00 g, 2.70 mmol, 1.00 equiv.), 1.63 g TBDMS–Cl (10.81 mmol, 4.00 equiv.), and 1.10 g of imidazole (16.21 mmol, 6.00 equiv.) were dissolved in 2.5 mL of pyridine and then stirred at 50 °C for 2 h under an argon atmosphere. At the end of the reaction time, the mixture was cooled to r.t. and quenched by addition of MeOH (2.0 mL) with continuous stirring. After 5 min, the product mixture was dissolved in 20 mL of CH_2Cl_2, added to aq. 5% HCl solution (10 mL), and extracted three times with CH_2Cl_2 (30 mL). The combined organic phases were washed with aq. 5% HCl solution (30 mL). The aqueous phases were extracted again with 30 mL of CH_2Cl_2. The combined organic phases were washed with saturated $NaHCO_3$ solution (40 mL) and brine (40 mL), and dried over $MgSO_4$. The solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (dichloromethane/EtOAc 20:1). The product was obtained as a white solid (1.77 g, 92%). ^{1}H NMR (400 MHz, chloroform-d): δ (ppm) = 8.21 (br. s, 1H), 8.05 (s, 1H), 6.02 (d, J = 6.6 Hz, 1H), 4.13–4.09 (m, 1H), 4.07–4.03 (m, 2H), 3.90 (dd, J = 11.6, 2.0 Hz, 1H), 3.74 (dd, J = 11.6, 2.0 Hz, 1H), 0.99 (s, 9H), 0.91 (s, 9H), 0.86 (s, 9H), 0.20 (s, 6H), 0.10 (s, 3H), 0.07 (s, 3H), 0.03 (s, 3H), −0.05 (s, 3H). HR-MS (ESI): calcd. for $C_{27}H_{53}IN_2O_6Si_3$ $[M]^+$: 712.22561; found $[M+H]^+$: 713.23203. The spectroscopic data agree with the literature.[32]

46: **42** (0.500 g, 701 µmol, 1.00 equiv.), 27.0 mg CuI (140 µmol, 0.20 equiv.), and 81.0 mg $Pd(PPh_3)4$ (70.1 µmol, 0.10 equiv.) were placed under an argon atmosphere and dissolved the starting in 5.00 mL of dry THF. Subsequently, 0,195 g **45** (1,05 mmol, 1.50 equiv.) and 389 µL Et_3N (0.284 g, 2.81 mmol, 4.00 equiv.) were added and the reaction mixture was stirred at 50 °C for 3 h. After removing the solvent under reduced pressure, the crude product was dissolved in EtOAc (50 mL), washed with 5% HCl solution (40 mL), saturated $NaHCO_3$ (40 mL) and brine (40 mL). The organic phase was dried over $MgSO_4$, filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (dichloromethane/EtOAc 20:1). The product was obtained as a yellow solid (0,412 g, 80%). ^{1}H NMR (400 MHz, chloroform-d): δ (ppm) = 8.13 (br. s, 1H), 8.10 (s, 1H), 5.99 (d, J = 5.8 Hz, 1H), 4.36 (d, J = 5.2 Hz, 2H), 4.13–4.09 (m, 1H), 4.05 (dd, J = 4.6, 1.7 Hz, 2H), 3.94 (dd, J = 11.6, 2.1 Hz, 1H), 3.75 (dd, J = 11.6, 1.6 Hz, 1H), 0.97 (s, 9H), 0.91 (s, 9H), 0.87 (s, 9H), 0.17 (d, J = 3.2 Hz, 6H), 0.09 (d, J = 7.3 Hz, 6H), 0.04 (s, 3H), −0.02 (s, 3H). ^{13}C NMR DEPT 135 (101 MHz, chloroform-d): δ (ppm) = 143.6, 88.4, 86.1, 76.3, 72.0, 62.7, 30.6, 26.2, 25.8, 25.7. HR-MS (ESI): calcd. for $C_{32}H_{56}F_3N_3O_7Si_3$ $[M]^+$: 735.33782; found $[M+H]^+$: 736.34470.

48: **46** (0.400 g, 0,543 mmol, 1.00 equiv.) was dissolved in 2.80 mL of MeOH. NaOH (0.130 g, 2.36 mmol, 6.00 equiv.) dissolved in 280 µL H_2O were added dropwise. The reaction mixture was stirred at r.t. for 2 h. The solvent was removed under reduced pressure and the residue was redissolved in 40 mL CH_2Cl_2. It was washed twice with brine (30 mL), dried over $MgSO_4$, filtered and the filtrate was concentrated under reduced pressure. The crude product was purified via column chromatography on silica gel (CH_2Cl_2/EtOAc 8:2 to CH_2Cl_2/MeOH 5%). The product was obtained as a pale brown solid (244 mg, 70%). ^{1}H NMR (400 MHz, chloroform-d): δ (ppm) = 7.99 (s, 1H), 5.99 (d, J = 5.8 Hz, 1H), 4.14–4.09 (m, 1H), 4.07–4.02 (m, 2H), 3.94 (dd, J = 11.7, 1.8 Hz, 1H), 3.75 (dd, J = 11.6, 1.6 Hz, 1H), 3.59 (s, 2H), 0.98 (s, 9H), 0.91 (s, 9H), 0.87 (s, 9H), 0.18 (d, J = 4.4 Hz, 6H), 0.09 (d, J = 7.1 Hz, 6H), 0.03 (s, 3H), −0.02 (s, 3H). ^{13}C NMR (101 MHz, chloroform-d): δ (ppm) = 161.4, 149.2, 142.5, 100.3, 95.3, 88.3, 86.2, 76.2, 73.5, 72.4, 63.0, 32.5, 26.4, 26.0, 25.8, 18.7, 18.2, 18.1, −4.25, −4.45, −4.59, −4.61, −5.20, −5.22. HR-MS (ESI): calcd. for $C_{30}H_{57}N_3O_6Si_3$ $[M]^+$: 639.35552; found $[M+H]^+$: 640.36273.

50: **48** (0.104 g, 0.163 mmol, 0.95 equiv.) was dissolved in 2.00 mL dry THF. 50.0 mg BCN-NHS ester **37** (0.172 µmol, 1.00 equiv.) and 95.0 µL triethylamine (70.0 mg, 0.687 mmol, 4.00 equiv.) were added. The reaction mixture was allowed to stir at r.t. for 19 h. The solvent was removed under reduced pressure and the residue was redissolved in CH_2Cl_2 (30 mL). It was washed with water (30 mL), $NaHCO_3$ solution (2 x 30 mL), and brine (30 mL). The organic layer was dried over $MgSO_4$, filtered and the filtrate was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc 4:1). The product was obtained as a pale-yellow solid (0.195 mg, 73%). ^{1}H NMR (400 MHz, chloroform-d) δ (ppm) = 8.16 (s, 1H), 8.03 (s, 1H), 5.98 (d, J = 5.9 Hz, 1H), 4.91 (s, 1H), 4.23–4.14 (m, 4H), 4.14–4.09 (m, 1H), 4.07–4.03 (m, 2H), 3.93 (dd, J = 11.6, 1.9 Hz, 1H), 3.75 (dd, J = 11.6, 1.6 Hz, 1H), 2.34–2.16 (m, 6H), 1.66–1.50 (m, 4H), 1.42–1.31 (m, 1H), 0.97 (s, 9H), 0.91 (s, 9H), 0.87 (s, 9H), 0.18 (d, J = 4.1 Hz, 6H), 0.08 (d, J = 7.3 Hz, 6H), 0.03 (s, 3H), −0.03 (s, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ (ppm) = 161.3, 151.2, 149.1, 143.2, 99.7, 99.0, 90.0, 88.3, 86.3, 76.3, 74.8, 72.4, 63.0, 31.9, 29.2, 26.3, 25.9, 25.8, 21.6, 20.3, 18.7, 18.2, 18.0, 17.9, -4.25, -4.45, -4.59, -4.62, −5.24. HR-MS (ESI): calcd. for $C_{41}H_{69}N_3O_8Si_3$ $[M]^+$: 815.43925; found $[M+H]^+$: 816.44682.

7: To a solution of 0,100 g **50** (0.123 mmol, 1.00 equiv.) in 2 mL dry THF, 0.160 mL triethylamine-trihydrofluoride (0.158 mmol, 0.980 mmol, 8.00 equiv.) were added. The reaction mixture was stirred at 50 °C for 16 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1). The product was obtained as an off-white solid (0.044 mg, 76%). ^{1}H NMR (400 MHz, methanol-d_4): δ (ppm) = 8.30 (s, 1H), 5.84 (d, J = 4.0 Hz, 1H), 4.19–4.09 (m, 4H), 4.06–4.01 (m, 2H), 4.01–3.96 (m, 1H), 3.84 (dd, J = 12.3, 2.6 Hz, 1H), 3.71 (dd, J = 12.3, 2.9 Hz, 1H), 2.26–2.08 (m, 6H), 1.63–1.50 (m, 2H), 1.40–1.28 (m, 1H), 0.96–0.85 (m, 2H). ^{13}C NMR (101 MHz, methanol-d_4): δ (ppm): 164.5, 158.8, 151.4, 145.5, 100.1, 99.5, 91.0, 90.7, 86.4, 76.0, 74.9, 71.0, 64.1, 61.9, 31.9, 30.2, 21.9, 21.4, 18.9. HR-MS (ESI): calcd. for $C_{23}H_{27}N_3O_8$ $[M]^+$: 473.17981; found $[M+H]^+$: 474.18738.

51: **49** (0.130 g, 0.202 mmol, 1.00 equiv.) and Et_3N (0.112 mL, 0.808 mmol, 4.00 equiv.) were dissolved in 5 mL dry DMF. BCN-NHS ester **38** (0.088 g, 0.303 mmol, 1.50 equiv.) was added and the reaction solution was stirred for 16 h at r.t. The solvent was removed under reduced pressure. The residue was dissolved in 20 mL MeOH and stirred with Amberlite IRA 402 bicarbonate for 30 min. After filtration and removal of the solvent under reduced presssure, the product was purified by column chromatography (CH_2Cl_2/MeOH = 50:1) to obtain a colorless foam (0.096 g, 57%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.43 (s, 1H), 7.39 (s, 1H), 7.12 (s, 1H), 5.87 (d, J = 7.5 Hz, 1H), 4.50 (d, J = 7.8 Hz, 1H), 4.33–4.21 (m, 2H), 4.12–4.04 (m, 4H), 3.95–3.90 (m, 1H), 3.16 (d, J = 5.2 Hz, 4H), 2.15 (d, J = 14.5 Hz, 8H), 1.57–1.48 (m, 4H), 1.25 (d, J = 10.3 Hz, 2H), 1.00–0.78 (m, 27H), 0.20–0.21 (m, 16H). HR-ESI-MS (m/z): $[M+H]^+$ calcd. for $C_{41}H_{74}N_3O_8Si_3^+$: 820.47782; found: 820.47751. The spectroscopic data are in agreement with the literature.[30]

6: **51** (0.078 g, 0.116 mmol, 1.00 equiv.) was dissolved in 5 mL THF_{dry} under argon atmosphere. $Et_3N{\cdot}3$ HF (0.567 mL, 1.163 mmol, 10.0 equiv.) was slowly added. The mixture was stirred at r.t. for 2 s. After completion of the reaction, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2/MeOH 6:1) to obtain a pinkish foam (0.028 g, 51%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.29 (s, 1H), 7.72 (s, 1H), 7.10 (t, J = 5.7 Hz, 1H), 5.77 (d, J = 5.5 Hz, 1H), 5.34 (d, J = 5.8 Hz, 1H), 5.12–5.03 (m, 2H), 4.04 (s, 1H), 3.97 (q, J = 4.7 Hz, 1H), 3.82 (q, J = 3.5 Hz, 1H), 3.67–3.52 (m, 3H), 2.96 (q, J = 6.7 Hz, 2H), 2.26–2.13 (m, 7H), 1.53 (dt, J = 14.8, 7.5 Hz, 4H), 1.27 (dt, J = 17.1, 8.4 Hz, 2H), 0.97–0.82 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ = 163.4, 156.4, 150.6, 136.6, 129.5, 113.0, 99.0, 87.6, 84.7, 73.3, 69.9,

61.3, 60.9, 28.6, 28.3, 23.7, 20.9, 19.5, 17.7, 13.9, 10.9. HR-ESI-MS (m/z): $[M+H]^+$ calcd. for $C_{23}H_{32}N_3O_8^+$: 478.21839; found: 478.21856.

Synthesis of oligonucleotides DNA1-DNA6

DNA1, DNA3 and DNA5: Oligonucleotides were synthesized by using standard solid-phase phosphoramidite synthesis protocol on a H-6 DNA/RNA synthesizer by K&A Laborgeräte. Phosphoramidites of 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, thymidine (Sigma Aldrich) were used as a 67 mM solution in acetonitrile. Phosphoramidites **40 a** or **40 b** were used as a 100 mM solution in CH_2Cl_2. As solid phase, CPG columns (1 μmol, Sigma Aldrich) were used. After synthesis, the CPG columns were dried in high vacuum. The CPG was removed from the columns and transferred to an Eppendorf reaction vial. 700 μL of 25% aq. NH_4OH was added and the suspension incubated over night at 55 °C, followed by removal of the solvents by vacuum centrifugation (35 min, 35 °C, 100 mbar, followed by 4 h, 25 °C, 0.100 mbar). The crude product was purified by a standard Glen Gel-Pak™ desalting protocol. MALDI-TOF with 3-HPA as a matrix substance: calcd. for **DNA1** $[M]^+$: 5213.92, found $[M+H]^+$: 5214.38; calcd. for **DNA3** $[M]^+$: 5231.91, found $[M+H]^+$: 5233.56; calcd. for **DNA5** $[M]^+$: 5209.89, found $[M+H]^+$: 5210.06;

DNA2, DNA4 and DNA6: Approximately 1 μmol of **DNA1**, **DNA3** and **DNA5** was dissolved in anhydrous DMSO (300 μL). NHS ester **38** (2 mg) was dissolved in anhydrous DMSO (100 μL) and added to the corresponding DNA solution. $(i\text{Pr})_2\text{NEt}$ (5 μL) was added and the vials were shook on a laboratory shaker for 16 h. After completion of the reaction, the solvent was removed in the vacuum centrifuge (8 h, 25 °C, 0.100 mbar). The DNA pellet was dissolved in water (600 μL) and purified via semi-preparative HPLC VDSpher OptiBio PUR 300 S18-SE column (250×10 mm, 5 μm, 300 μL injection, 0–20% acetonitrile, 0.1 M NH_4OAc, 40 °C, 30 min). The detection wavelength was set to 260 and 280 nm. MALDI-TOF with 3-HPA as a matrix substance: calcd. for **DNA2** $[M]^+$: 5390.04, found $[M+H]^+$: 5393.02; calcd. for **DNA4** $[M]^+$: 5408.12, found $[M+H]^+$: 5408.02; calcd. for **DNA6** $[M]^+$: 5385.97, found $[M+H]^+$: 5389.35. For annealing, the modified oligonucleotide (10 μM) was heated together with 1.20 equiv. of the corresponding complementary oligonucleotide in 10 mM NaP_i buffer (pH 7) and 250 mM NaCl solution to 90 °C for 10 min and then slowly cooled to r.t.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: bicyclononyne · click chemistry · Diels-Alder reaction · fluorescence · tetrazine

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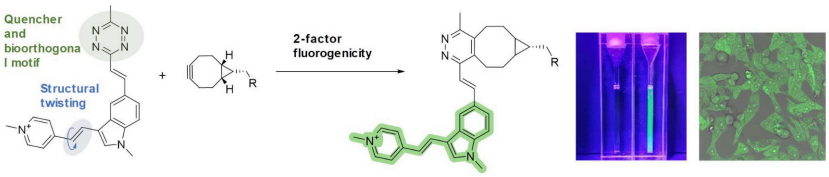

Fast and bright! The inverse electron demand Diels-Alder reactions of the synthetic tetrazine-modified dyes with bicyclononyne-modified DNA follow the new concept of the two-factor fluorescence fluorogenicity which improves the signal-to-noise ratio such that washing procedures prior to cell imaging are not needed. The labelling reactions proceed also extremely fast.

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Two-Factor Fluorogenicity of Tetrazine-Modified Cyanine-Styryl Dyes for Bioorthogonal Labelling of DNA