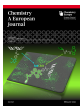

Fast and Efficient Postsynthetic DNA Labeling in Cells by Means of Strain-Promoted Sydnone-Alkyne Cycloadditions

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Abstract: Sydnones are highly stable mesoionic 1,3-dipoles that react with cyclooctynes through strain-promoted sydnone-alkyne cycloaddition (SPSAC). Although sydnones have been shown to be valuable bioorthogonal chemical reporters for the labeling of proteins and complex glycans, nucleic acids have not yet been tagged by SPSAC. Evaluation of SPSAC kinetics with model substrates showed fast reactions with cyclooctyne probes (up to $k = 0.59\ M^{-1}s^{-1}$), and two different sydnones were effectively incorporated into both 2′-deoxyuridines at position 5, and 7-deaza-2′-deoxyadenosines at position 7. These modified nucleosides were synthetically incorporated into single-stranded DNAs, which were successfully postsynthetically labeled with cyclooctyne probes both in vitro and in cells. These results show that sydnones are versatile bioorthogonal tags and have the premise to become essential tools for tracking DNA and potentially RNA in living cells.

Over the past decade, bioorthogonal chemical ligations have emerged as cornerstone technologies for the study of proteins, glycans and nucleic acids in cells and organisms.[1] Among the various reported bioorthogonal chemistry, strain-promoted alkyne–azide cycloadditions (SPAAC),[2] inverse electron-demand Diels-Alder reactions (iEDDA)[3] or photoinduced cycloadditions between tetrazoles and alkenes[4] have the advantage of alleviating the need for potentially cytotoxic metal species. In order to solve the stability shortcomings of previous bioorthogonal reagents (i.e., azides can be subject to reduction in biological systems[5]), we and others have recently demonstrated that sydnones, highly stable mesoionic 1,3-dipoles, can react with cyclooctynes through strain-promoted sydnone-alkyne cycloaddition (SPSAC), for the labeling of proteins[6] and complex glycans.[5] Attractive features of sydnones include high stability in biological media, synthetic modularity for tunable reactivity, and photo-activation for spatial and temporal control of their chemical reactivity.[7] Moreover, sydnones have also been shown to be excellent fluorogenic reagents allowing for the visualization of biomolecules with exceptional signal to background ratio.[6d,8]

The iEDDA reaction has been tremendously employed for the biorthogonal labeling of proteins, in particular between tetrazine-modified amino acid and labels bearing either strained alkenes[3c,9] or the strained alkyne bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN), mostly due to its fast reactivity. The second-order rate constants of BCN with tetrazines lie in the range of $k = 1–10^3\ M^{-1}s^{-1}$.[10] However, iEDDA reactions with BCN have been shown to be poorly suited for nucleic acid labeling since tetrazines-modified DNA only exhibit 35–40% labeling yields (Figure 1).[11] Triazines are more stable,[12] but react slower with $k \leq 10^{-2}\ M^{-1}s^{-1}$.[12a,13] Triazine-modified DNA can therefore be labeled in good yields, but needs high concentration of the BCN-modified dyes as reactive counterparts.[14] Due to their fast

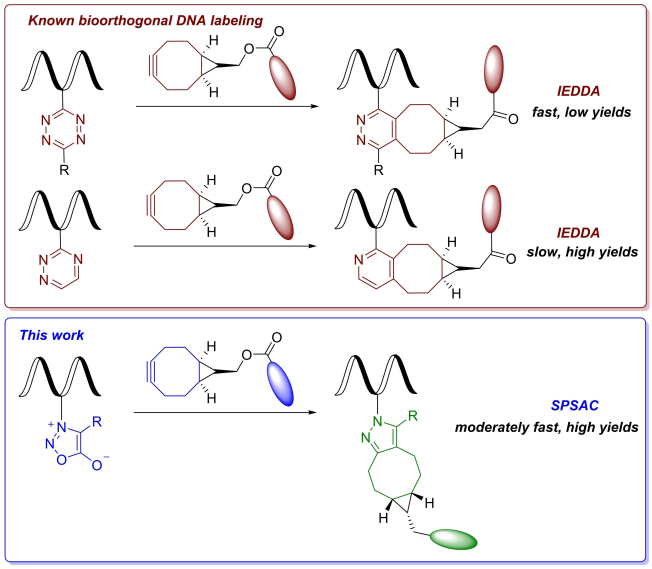

Figure 1. Postsynthetic labeling of DNA by iEDDA using tetrazines and triazines as reactive groups in comparison with SPSAC.

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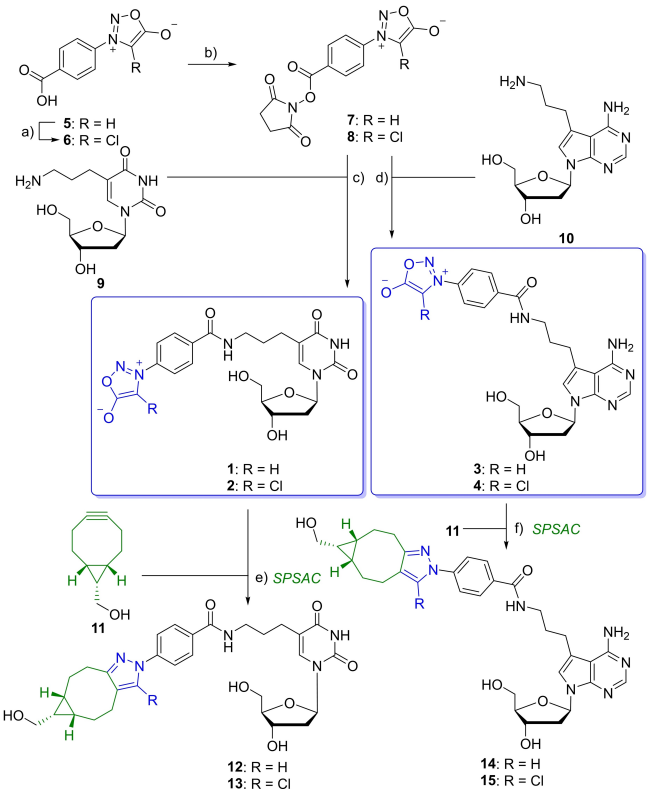
kinetics and successful application for the labeling of proteins and complex glycans, SPSACs provide an important alternative to the currently limited iEDDA labeling of nucleic acids labeling. Herein, we present the synthesis of four different 2′-deoxynucleosides **1**–**4** modified with sydnones, their SPSAC reactivity with cyclooctyne probes, including the compatibility of sydnone reporters with oligonucleotides and the postsynthetic labeling of DNA in vitro and in fixed cells.

First, we investigated the reactivity of two classical sydnones, 3-phenylsydnone (PhSydH) and 4-chloro-3-phenylsydnone (PhSydCl), chosen for their excellent stability in biological environments, with three representative cyclooctynes commonly used in bioconjugation-MeO-DIBO,[15] MeO-DIBAC[16] and BCN[17] (Figure 2). The second-order rate constants of cycloadditions were determined by monitoring the formation of the pyrazole product by ^{1}H NMR spectroscopy in CD_3OD at 25 °C (Figures S1–S6 in the Supporting Information). While 3-phenylsydnone reacted rather sluggishly with MeO-DIBO and BCN, its rate constant with MeO-DIBAC ($k = 0.191\ M^{-1}s^{-1}$) was on par with the cycloaddition of DIBAC with azides.[18] Additionally, introduction of a chlorine atom on C-4 of the mesoionic ring further enhanced the reactivity of the sydnone with all employed cyclooctynes. For instance, 4-chloro-3-phenylsydnone reacted twice faster than the unchlorinated analogue with the dibenzylic probes and a remarkable 30 times faster with the less sterically hindered BCN. The most rapid cycloaddition was observed between the BCN probe and 4-chlorosydnone at a rate constant of 0.57 $M^{-1}s^{-1}$.

To investigate the potential impact of the presence of the nucleoside on the SPSAC reactivity, sydnone-modified nucleosides **1**–**4** were prepared in a straightforward way (Scheme 1).



Scheme 1. Synthesis of sydnone-modified 2′-deoxyuridines **1** and **2** and the 7-deaza-2′-deoxyadenosine **3** and **4** and SPSACs with BCN **11**: a) NaClO (12% act. Cl_2), dioxane/HCl, RT, 20 h, 43%; b) *N*-hydroxysuccinimide, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, DMF, RT, 16 h, **5**: 78%, **6**: 89%; c) Et_3N, DMF, RT, 16 h, **1**: 29%, **2**: 54%; d) Et_3N, DMF, RT, 16 h, **3**: 29%, **4**: 36%; e) MeOH, 20 °C, 4 h; f) MeOH, 20 °C, 4 h.

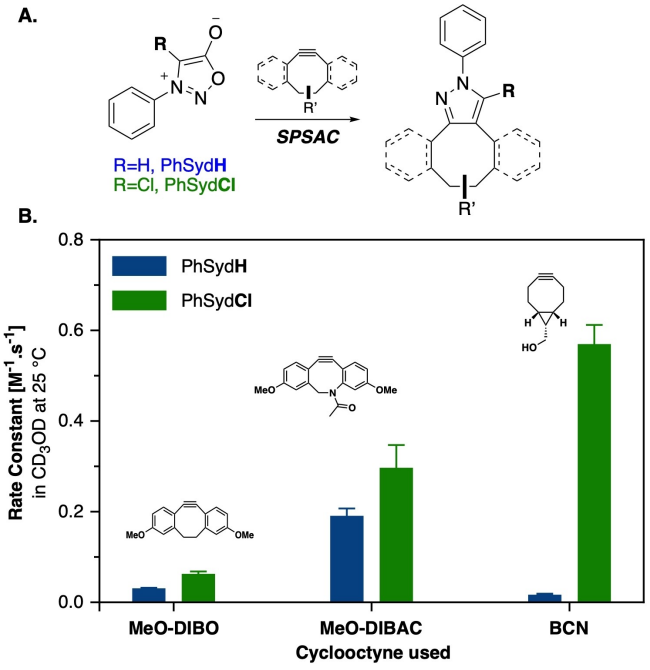


Figure 2. Second-order rate constants of the strain-promoted sydnone–alkyne cycloadditions (SPSAC) between sydnones PhSydH and PhSydCl with three cyclooctynes (MeO-DIBO, MeO-DIBAC and BCN).

Briefly, sydnones **5** and **6** were activated as NHS-esters **7** and **8** according to standard procedures and coupled to the 2′-deoxyuridine **9** and the 7-deaza-2′-deoxyadenosine **10** in a classical NHS-coupling protocol. **9** and **10** are broadly applied DNA modifiers and were chosen to have a flexible linker between the sydnone moiety and the DNA core (Figures S16–S36).[8a,19]

The model sydnone-modified nucleosides **1** and **2** were then labeled with BCN (**11**) and the SPSACs were followed by UV/Vis absorbance changes (Figure 3). After addition of 10 equiv. of BCN (**11**), the characteristic sydnone absorbances at around 325 nm decreased, confirming consumption of the starting sydnones **1** and **2**. Concomitantly, new bands appeared that were assigned to the pyrazole products (Figure 3, ca. 300 nm for product **12** (top) and 270 nm for product **13** (bottom)). Both products were identified by MALDI-TOF mass spectrometry (Figures S37 and S38). In good agreement with our initial kinetic study (Figure 2), the chlorinated sydnone-nucleoside **2** reacted significantly faster than unsubstituted sydnone **1**, as complete consumption of **2** was achieved within 240 min, while only approximately 50% of **1** was converted. Remarkably, the UV/Vis absorbances recorded during both reactions showed isosbestic points at 272/314 nm (for **1** to **12**) and at 252/299 nm (for **2** to **13**), highlighting that the SPSAC

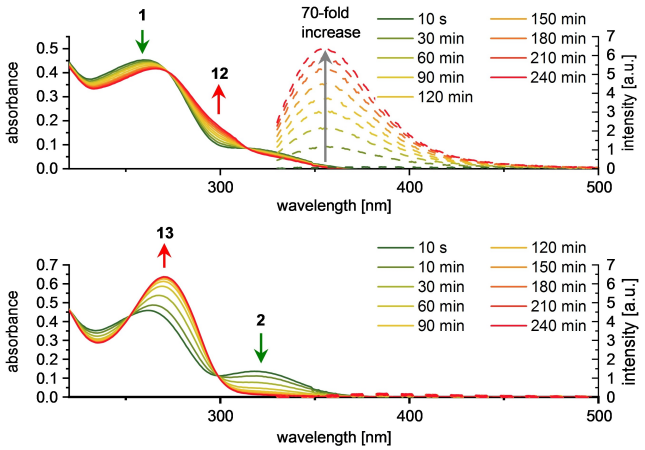
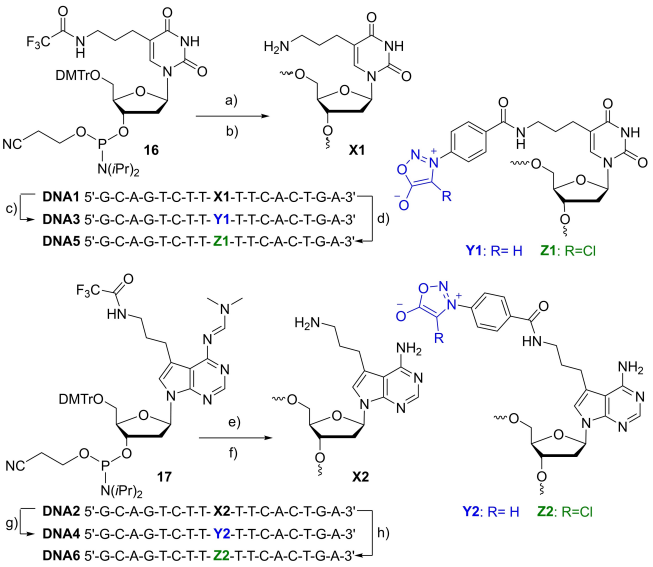


Figure 3. UV/Vis absorbance (solid lines) and fluorescence (dashed lines, $\lambda_{ex} = 315$ nm) changes recorded during SPSAC labeling experiments with 25 μM nucleoside **1** (top) or nucleoside **2** (bottom) and 250 μM BCN **11** (10.0 equiv.) in MeOH at 20 °C over 4 h. For experiments with nucleoside **3** and nucleoside **4**, see Figures S14 and S15.



Scheme 2. Synthesis of sydnone-modified **DNA3**–**DNA6**: a) DNA synthesizer; b) 25% NH_4OH, 55 °C, 16 h; c) **7**, Et_3N, DMSO, RT, 16 h d) **8**, Et_3N, DMSO, RT, 16 h; e) DNA synthesizer; f) 25% NH_4OH, 55 °C, 16 h; g) **7**, Et_3N, DMSO, RT, 16 h; f) **8**, Et_3N, DMSO, RT, 16 h.

reactions of nucleosides **1** and **2** generated very cleanly the desired conjugated products **12** and **13**, respectively, without any intermediates. Moreover, the formation of conjugate **12** was accompanied by a 70-fold increase of fluorescence intensity, an interesting feature for future fluorogenic labeling. To evaluate the generality of the SPSAC labeling protocol, we also investigated the reaction of BCN (**11**) with the sydnone modified adenosine-based nucleosides **3** and **4** by UV/Vis absorbance (Figures S14, S15, S40, and S41). Gratifyingly, BCN (**11**) labeled the nucleosides **3** and **4** as efficiently as **1** and **2**, respectively. The kinetics (Figures S46–S55) revealed second-order rate constants of $k = 0.07 \pm 0.013\ M^{-1}s^{-1}$ for **3** and $4.4 \pm 1.5\ M^{-1}s^{-1}$ for **4**. The difference in reactivity between the non-chlorinated and chlorinated sydnone is stronger as nucleoside modifications. In order to diversify our cyclooctyne probe, the experiments were also conducted with **1** and **2** in presence of a DIBAC-amine as a reactive partner. Due to strong spectral overlap, the kinetics could not be resolved by following the absorbance changes. However, successful formation of the desired pyrazoles was confirmed by MALDI-TOF mass spectrometry (see Figure S39 for the adduct between **1** and DIBAC-amine).

To investigate the applicability of using SPSAC-based ligation of more complex oligonucleotides, we introduced the sydnone moieties on oligonucleotides using a straightforward postsynthetic approach employing phosphoramidite **16** and **17**. Briefly, **16** and **17** were first synthesized according to literature,[20] and incorporated into oligonucleotide by standard solid-phase and automated DNA synthesis (Scheme 2). Subsequent treatment with aqueous ammonium hydroxide cleaved off the TFA protecting group and yielded the oligonucleotides bearing a reactive amine (**DNA1** and **DNA2**). **DNA1** and **DNA2** were then reacted with the NHS esters **7** and **8** to give the sydnone-modified strands **DNA3**–**DNA6**, which were purified by RP-HPLC and characterized by MALDI-TOF mass spectrometry (Figures S31–S36). Moreover, the UV/Vis absorption of **DNA3**–**DNA6** further confirmed the attachment of a sydnone moiety with the presence of a characteristic band between 310 and 350 nm.

Next, SPSAC labeling of DNA strands **DNA3**–**DNA6** was performed with DIBAC conjugated with a fluorophore, to mimic more closely future biological applications in cells. To note, DIBAC was chosen instead of BCN, as it is a more versatile cyclooctyne for SPSAC since it reacts equally fast with both unsubstituted and chloro-substituted sydnones **3** and **4**, and more fluorescently labeled DIBAC reagents for cellular applications are commercially available as compared to BCN. Accordingly, **DNA3** was reacted with 10.0 equiv. of Cy3-DIBAC in aqueous solution (containing 5% DMSO) at room temperature and the reaction was followed by HPLC analysis (Figure S7)Surprisingly, full conversion of **DNA3** ($t_R = 9.5$ min) to **DNA3**-Cy3 ($t_R = 22$ min) was achieved in only ~1 h of reaction; this is significantly faster than the reaction of nucleoside **1** with BCN. Even upon immediate injection following the addition of Cy3-DIBAC (~3 min reaction time associated with sample preparation and injection), significant product formation was detected. This underscores the fast kinetics of the SPSAC between sydnone-modified oligonucleotides and DIBAC. Also, it is possible that the reaction was further accelerated by the use of water instead of methanol as solvent.[21] The ragged shape of the product signal indicates the formation of the two possible regioisomers of the reaction, as MALDI-TOF mass spectrometry shows only the correct product mass. To further challenge the SPSAC labeling, the experiments were performed with only 1.50 equiv. of Cy3-DIBAC. The reaction depicted similarly clean HPLC chromatograms but required, as expected, longer reaction time to reach completion (~3 h; Figure S8). **DNA5** was also labeled with 1.50 equiv. of Cy3-DIBAC and HPLC analysis

(Figure S9) indicated significant product formation after only ~3 min reaction time, and full conversion was reached after ~1 h of incubation. The SPSACs with **DNA4** and **DNA6** were comparably fast in the presence of 1.5 equiv. Cy3-DIBAC (Figures S10 and S12). The reactions of **DNA4** and **DNA6** with 5.0 equiv. Cy3-DIBAC were completed in 2 min (Figures S11 and S13). Due to this very fast reaction, we could not determine the second-order rate constants for the sydnone-modified DNA. Taken together, these results indicate that complex sydnone-modified oligonucleotides can be labeled cleanly and efficiently in aqueous media at room temperature with the cyclooctyne DIBAC (MALDI-TOF MS analysis in Figures S42–S45).

Finally, SPSAC was applied for the labeling of DNA in cells. Accordingly, HeLa cells were transfected with 0.7 μM of oligonucleotides **DNA3** or **DNA5**, fixed using 4% paraformaldehyde solution, treated with 7 nM of sulfoCy3-DIBAC for 3 h, washed and analyzed by confocal fluorescence microscopy. Gratifyingly, significant fluorescence staining was clearly observed in the endosomes and the cytosol of the cells, when **DNA3** and **DNA5** were employed, while the control experiment, without any transfected DNA, only displayed negligible fluorescence (Figure 4). This result illustrates that oligonucleotides containing a sydnone bioorthogonal tag can be efficiently and selectively labeled in complex biological systems with the cyclooctyne DIBAC, opening exciting possibilities for in-cell imaging.

To conclude, we have reported the first examples of strain-promoted sydnone-alkyne cycloaddition-based labeling of nucleosides and oligonucleotides. Although the dual postsynthetic strategy of amide coupling of the sydnone to DNA and subsequent SPSAC might seem cumbersome, it was chosen to highlight that sydnones, as bioorthogonal tags, are compatible with both nucleoside and oligonucleotide chemistry. In addition, the labeling of sydnone-modified oligonucleotides with various cyclooctyne probes by SPSAC proved to be particularly clean and efficient in aqueous media, notably between the chlorosydnone-oligonucleotide and DIBAC. Additionally, HeLa cells, previously transfected with sydnone-modified oligonucleotides, were successfully labeled by using SPSAC bioorthogonal ligation. Sydnones react more slowly in SPSACs than tetrazines in inverse electron demand Diels-Alder reactions, but, unlike tetrazines, are compatible with DNA labeling and lead to quantitative yields. In addition, although triazines are also stable and give DNA labeling yields of $>80\%$,[12,14] they react more slowly with BCN than sydnones.[12a,13] Finally, strain-promoted azide–alkyne cycloadditions are generally slower than SPSACs. This study demonstrates that sydnones are versatile bioorthogonal tags that react rather quickly and in high yields; this makes them an excellent compromise between stability and reactivity. Thus, sydnones have the potential to become essential tools for tracking DNA and possibly RNA in cells.

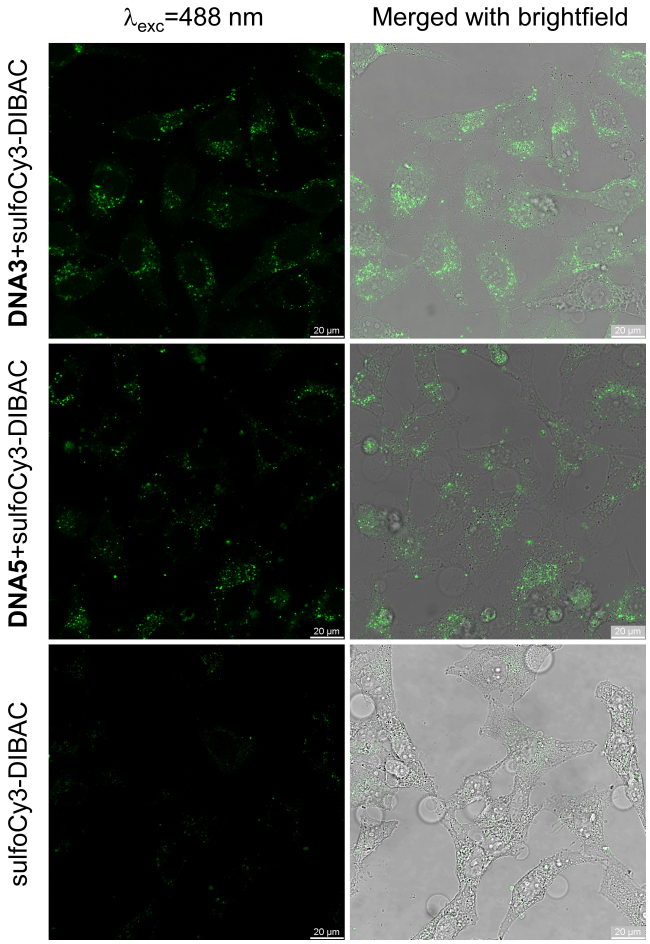


Figure 4. Confocal laser microscopy images of HeLa cells that were transfected for 26 h with either 0.7 μM of **DNA3** or **DNA5** and subsequently fixed and labeled by means of SPSAC with sulfoCy3-DIBAC (7 nM) within 4 h. The cells were washed with 2× PBS, followed by imaging. $\lambda_{ex}=488$ nm, $\lambda_{em}=550$–650 nm. The merged column presents the merger of the fluorescence and the transmission.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: cell biology · cyclooctyne · fluorescence · oligonucleotide · sydnone · strain

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