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View Article Online
DOI: 10.1039/C9CC02882J

COMMUNICATION

Photo-accelerated "Click" Reaction between Diarylsydnone and Ring-strained Alkyne for Bioorthogonal Ligation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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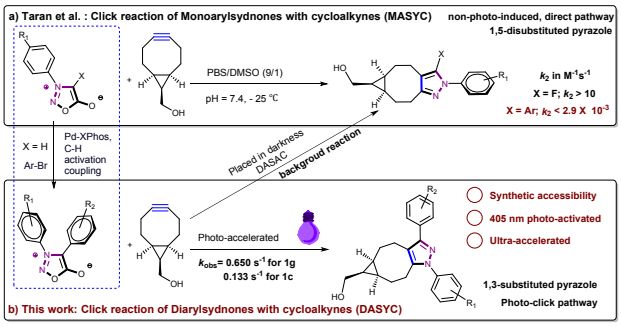
We constructed a library of diarylsydnone (DASyd) candidates in search of a photoclickable reaction toward alkynes, enabling an ultra-accelerated reactivity, while suppressing the background cycloaddition in dark. The *in-vitro* and *in-vivo* protein labelling experiments revealed that the photo-accelerated DASyd-alkyne cycloaddition exhibit robust selectivity.

Bioorthogonal reactions are elaborate chemical tools of high fidelity, not only for probing the dynamics but also functionalization of biomolecules in their natural environment.[1] In the past two decades, plenty of efforts have been made to enrich the library of available bioorthogonal strategies meeting the stringent requirements, including: nitrone-alkyne cycloaddition,[2] traceless Staudinger ligation,[3] tetrazine-dienophiles,[4] Cu-catalyzed[5] or Cu-free "azide-alkyne" ligation,[6] etc. In particular, the photo-click reactions,[7] such as nitrile imine (**NI**) mediated tetrazole-[8] and sydnone-alkene[9] conjugation, due to their smart response and high spatiotemporal controllability, have become an appealing research tool for ultra-fine ligation purpose. Although there are several photo-click cycloadditions that could be utilized selectively for olefins targeting ligation, like: photo-IEDDA,[10] PQA,[11] the study of alkyne centered strategy is still in infancy. The photo-decarbonylation of dibenzocyclooctyne (DIBO)[12] had been investigated by Popik et al. for bioconjugation, but the relatively slow ligation rate retarded its use under ultra-diluted condition.[13] Therefore, the exploration of new photo-initiated or photo-accelerated alkyne cycloaddition is still an intriguing yet challenging task to harness superior manipulation of the reaction process in 3-dimensional space.

Since Taran et al. reported the "Cu-catalyzed" regioselective click reaction of monoarylsydnone (MASyd)-alkyne cycloaddition,[14] sydnone, an heterocyclic mesoionic dipole,[15] had been extensively explored for bio-conjugation featuring



Scheme 1. Comparison of photo-activation versus DASAC strategy.

with both easy accessibility and decent stability. The "Cu-free" version was achieved via ring-strain promotion with the reaction rate up to 0.054 $M^{-1}s^{-1}$ and 1.46 $M^{-1}s^{-1}$ via the use of bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN) by Chin,[16] and dibenzoazacyclooctyne (DIBAC) by Murphy,[17] respectively. Recently, a significant advance has emerged as a flash ligation with the rate up to 10^4 $M^{-1}s^{-1}$, between halogen-substituted MASyd and ultra-strained 3,3,6,6-tetramethylthiacycloheptyne (TMTH) or BCN (SPSAC, Scheme 1), reported by Taran's team.[18] Further developments of MASyd were accomplished in 2018 and 2019 by Friscourt[19] and Taran,[20] respectively, mainly focusing on its utilization as biochemical tools affording monoarylpyrazole. Nevertheless, due to the lack of promising photo-reactivity of the MASyd, the utilization of this approach is diminished in spatiotemporal maneuverability demanding task where visible-light activation is preferred to reduce the photo-toxicity, gain higher tissue penetration.

In light of the **NI** mediated photo-click reaction of DASyd-alkene,[9] we proposed the alkene could be replaced with highly strained alkynes to harness an unexpected ligation-reactivity. Drastically different from the planar MASyd, the adjacent *ortho*-diaryl moieties on the DASyd exhibit a double-twisted conformation around its core, which is extremely detrimental toward the direct thermo-favored [3+2] pathway, hindering the attacking on the π-orbital of the mesoionic aromatics (Fig. S3a ESI†).[21] Under irradiation, the rapid depletion of DASyd via photoconversion into NI also constitutes a disadvantage toward undesired direct DASyd-alkyne cycloaddition (DASAC),

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†Electronic Supplementary Information (ESI) available: Containing details on experimental procedures, spectra property, characterization of all new compounds and XRD analysis. See DOI: 10.1039/x0xx00000x

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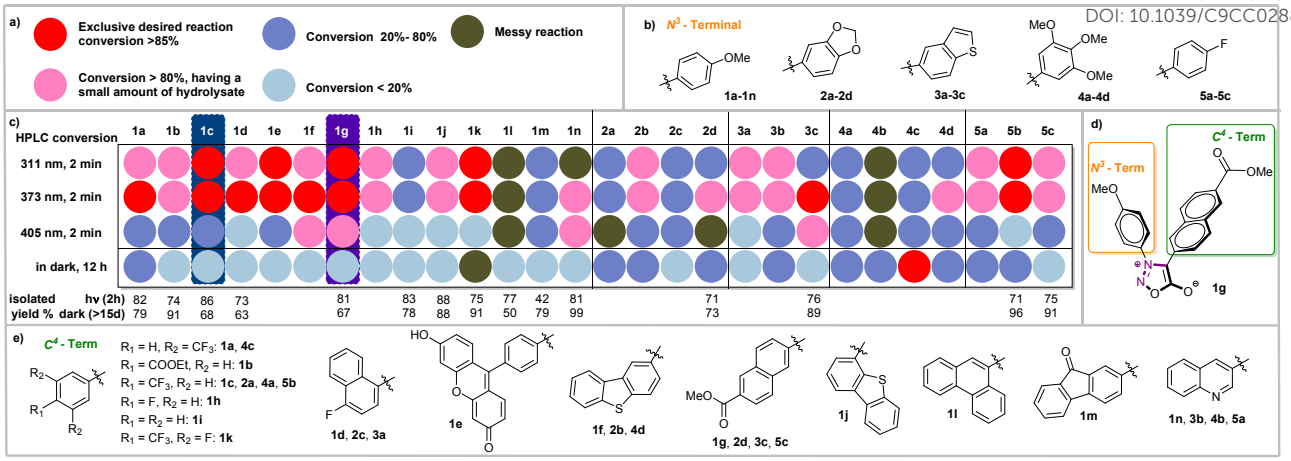


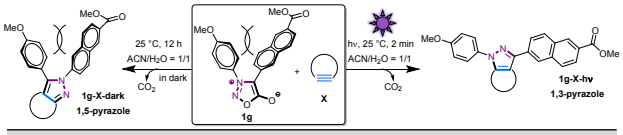
Fig. 1 Screening of DASyd-BCN combinations for photo-accelerated or non-photo-induced reaction. Experiments were carried out at 25 °C in acetonitrile (ACN)/H_2O = 1/1, irradiated with indicated light sources. [DASyd] = 100 μM, [BCN] = 500 μM. a) The colour codes for ligation characteristic analysed via HPLC-MS. b) The N^3 moieties in this study. c) Screening results. d) Structures of **1g** to illustrate the aryl building blocks on N^3 and C^4 moieties. e) The C^4 moieties involved.

forming 1,5-diarylpyrazole. In addition, the mechanism of photo-activation strategy was completely different from the DASAC pathway (Fig. S3a ESI†).[21] To demonstrate our hypothesis, an elaborate library of DASyd was constructed via C-H activation coupling[22] of MASyd (Scheme 1), placing electron rich aryls on N^3-terminal and chromophores on C^4-terminal to tune their optical properties.

For the goal of identifying a fast photo-responsive DASyd with minimal background reaction with BCN, a total of 112 arrays were performed under illumination with various light sources (311 nm hand-held lamp, 373 and 405 nm LED arrays) versus placing in dark for 12 h (Fig. 1 and Table S1 ESI†). The electron-tuning chromophores bearing on the C^4 (Fig. 1e) paired with electron-donating aryls (EDG) on N^3 (Fig. 1b) of DASyds would be preferred. Through coupling of featured aryl substituents (**1a**-**1n**) at C^4 on N^3-*p*-methoxyphenyl MASyd, DASyds **1f**, **1g** and **1n**, showed good conversions reacting with BCN at 405 nm light, but side-reactions were also observed for **1f** and **1n** at 311 nm. Under 311 nm irradiation, DASyd **1c** was a relatively good choice because **1k** and **1e** were expensive despite the fluorescent property of the later one. 'Scaffold hopping' at the N^3 position offers opportunities to elevate the photo-reactivity. However, DASyds (**2a**-**2d**), with a piperonyl moiety at N^3 position, lead to many side-reactions. Installation of a benzothiophen-5-yl at N^3 (**3a**-**3c**) didn't provide a remarkable bathochromic shift in their absorption (Table S5 ESI†), accompanied with unsatisfactory photo-conversion in UV range. With a 3,4,5-trimethoxyphenyl at N^3 (**4a**-**4d**), the overall photo-reactivity was decreased, but the DASAC was completed within 9 h for DASyd **4c** with a 3,5-$(CF_3)_2$phenyl at C^4. Lastly, 4-fluorophenyl (**5a**-**5c**) suppressed the photo-conversion, but slightly increased the rate of the DASAC pathway. Characterization of 1,5-diaryl (Table S7 ESI†) and 1,3-diaryl pyrazoles validated corresponding ligation pathways of non-photo-cycloaddition vs. photo-transformation, respectively. These results indicated DASyds **1g** shows negligible background reaction within 2 min temporal scale and is suitable for subsequent exploration.

To investigate the influence of the dipolarophiles alkyne (**a**-**e**), the cycloaddition reaction were also performed for **1g** and **1c** toward phenyl acetylene, electron-deficient EdU[23] as well as ring-strained DIBO,[24] BCN,[25] and TMTH (Tables 1 and S8 ESI†).[26] Unfortunately, terminal alkynes **a** and **b** reacted inertly under either photo-accelerated or dark condition with only trace amount of product detected (Figs. S17-19 ESI†). The strained alkynes, distinctly, displayed a significant improvement. Although, the yield for ligation of **1g** with DIBO was only 56.0% in comparison to 90.3% with BCN (Table 1, entry **c** vs. **d**), the DASAC-ligation of DIBO was also 1.4 times slower than that of BCN. The non-photo-controlled reaction of highly strained THTM was relatively fast (Table 1, entry **e**), consistent with Taran's report.[18] But, its mediocre photo-yield under 405 nm irradiation made it become inadequate for further study. Overall, as a light-accelerated approach, the BCN showed obvious advantages over the other alkynes (Tables 1 and S8, Figs. S17-S18 ESI†), ligated efficiently with **1g** under

Table 1. Photo-activation vs. DASAC reaction in dark for 1,3-Dipolar Cycloaddition of **1g** with various alkynes[a]



Entry	Alkyne	311 nm	373 nm	405 nm	Dark 120 s/12 h
a	PhAce	trace	trace	trace	N.D.[b]
b	EdU	trace	trace	trace	N.D.[b]
c	DIBO	53.7[c]	96.8	56.0[c]	N.D./8.60[c]
d	BCN	88.6[c]	97.2	90.3[c,d]	N.D./11.6[c]
e	TMTH	86.6	92.8	55.0[c,e]	21.8/92.7

[a]100 μM DASyd and 500 μM alkyne in ACN/H_2O (1/1) was irradiated with corresponding light sources for 120 s. Yields were determined by calibration curve. See Table S8 and Figs. S17-S18 in ESI† for details. [b]N.D. = not detected. [c]Incomplete transformation of DASyd. The DASAC adduct was observed in [d]trace or [e]7.75% yield.

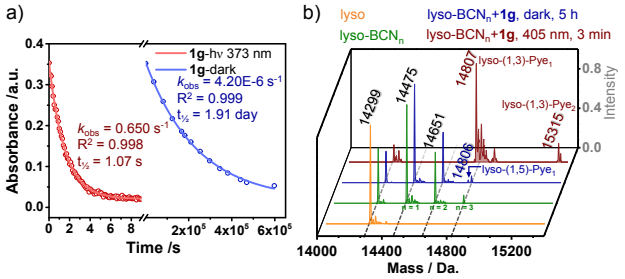


Fig. 2 Comparison of apparent kinetics and *in-vitro* protein labelling under irradiation versus in dark for **1g** with BCN. a) Real-time monitoring of absorption evolution, and exponential decay fitting results, 20 μM of **1g**, 1 mM of BCN in ACN/H_2O = 1/1; b) Deconvoluted HPLC-MS spectra for chemical modification of lysozyme and subsequent comparison of labelling efficiency, 5 μM of lysozyme, 20 eq. of **1g** in phosphate buffer (PB, pH = 6.0).

405 nm irradiation with undetectable DASAC (Table 1, entry **d**). Besides, the synthetic availability and compact size of BCN facilitate our research on its fast bioorthogonal applications.

Preliminary study on photo-accelerated cycloaddition kinetics ($k_{obs\text{-}p}$, the exponential decay) was obtained via tracing time-resolved absorbance evolution for DASyd **1g** at 350 nm and **1c** at 335 nm (λ_{max} in 250-400 nm range, but faint absorbance of the product) (Fig. S2 ESI†). The apparent kinetics were determined to be 0.65 ± 0.010 and 0.13 ± 0.002 s^{-1} (the [3+2] cycloaddition step, k_2 = 3.3 × 10^4 $M^{-1}s^{-1}$, Fig. S4 ESI†), respectively (Figs. 2a and S3b-3c ESI†), which were independent neither on the illumination intensity nor the concentration of BCN.[9] In contrast, the $k_{obs\text{-}t}$ for non-photo-induced reaction was also examined under identical concentration of reactants, DASyd/BCN = 1/50, (**1g** and **1c**, 20 μM), resulting in only 4.2 ± 0.17 × 10^{-6} and 3.9 ± 0.51 × 10^{-6} s^{-1} (k_2 = 4.9 × 10^{-3} $M^{-1}s^{-1}$, Fig. S3d ESI†), which was about 5 orders of magnitude slower than that of the photo-ligation reaction (Figs. 2a and S3b ESI†). Varying the BCN concentration, the $k_{obs\text{-}t}$ responded in positively linear correlation, proving that the DASAC-ligation was rate-limited by the initial [3+2] cycloaddition step (Fig. S3d ESI†).[18] Furthermore, the photo-conversion quantum yields of **1g** and **1c** were measured to be 0.24 (373 nm) and 0.21 (373 nm), respectively (Fig. S6 ESI†).

The *in-vitro* protein labelling experiment were conducted via a chemically tagged lysozyme-BCN$_n$ with DASyd **1g** (Fig. S9 ESI†). Comparing the efficiency of **1g** under 405 nm LED vs. in dark condition, the results showed that Lyso-BCN$_n$ was completely transformed into corresponding Lyso-(1,3-pyrazole)$_n$ within 3 min, while less than 2% into Lyso-(1,5-pyrazole)$_n$ for 5 h (Figs. 2b and S10 ESI†), respectively. The results of sharp contrast inspired us to investigate DASyd-BCN photoclick ligation under more stringent conditions.

To demonstrate the utility of DASyd-BCN photoclick ligation, allowing visualization of covalent modification of Lyso-(BCN)$_n$ on SDS-PAGE, a bifunctional Biotin-suflo-Cy3-DASyd (**6a**, Fig 3c)[27] was synthesized from **1g**. Protein labelling experiments were performed at 5 μM concentration. From the in-gel fluorescence imaging, it can be clearly seen that the exclusively labelling band of Lyso-pye$_n$ was detected in the lane where all the essential requirement for 405 nm photo-click was fulfilled in either PB buffer (Fig. 3a) or *E. coli* lysate (Fig. 3b). The weak

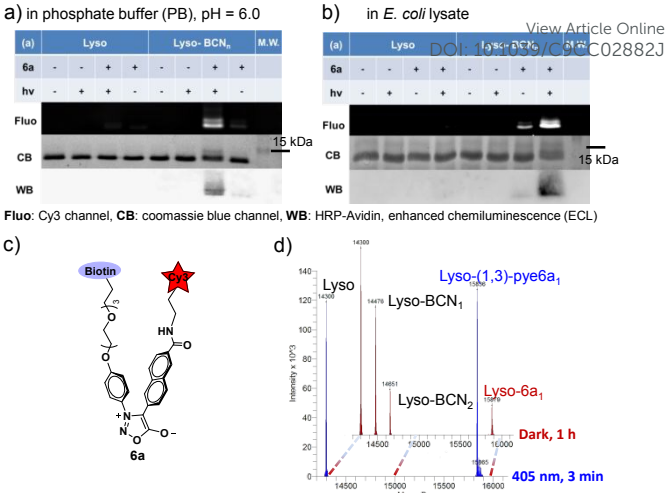


Fig. 3 Synthesis and application of Biotin-suflo-Cy3-DASyd **6a**. All protein labelling experiments were performed at 5 μM of Lyso-(BCN)$_n$, 20 eq. **6a**, 3 min. a) Images of SDS-PAGE and Western Blot assay for selective Lyso-(BCN)$_n$-**6a** photo-ligation in various channel were performed in PB buffer, b) or *E. coli* lysate. c) The structure of **6a**. d) Deconvoluted mass spectra for analysis of the photo-acceleration vs. cycloaddition in dark.

signal band from unexposed lane was caused by 1 h incubation and denaturation process at 80 °C (Figs. 3b and S11-S12 ESI†), leading to background cycloadduct. The selectivity of the photo-ligation was also confirmed via biotin targeting Western Blot assay in imaging through ECL channel and deconvoluted HPLC-MS analyses with exclusively matched mass (Fig. 3d). Collectively, all these data strongly supported the unique DASyd-BCN photo-reactivity with high specificity toward BCN reporter on proteins via the photo-pathway (Figs. 3 and S11-S12 ESI†).

To introduce the BCN reporter onto live cells, we modified the chimeric antibody Cetuximab with BCN-OCOO-PNP (Fig. S13 ESI†) and incubated the resulting Cetu-BCN with EGFR positive cancer cells, A549, through the specific binding on the outer membrane surface (Fig. 4a).[11] Because the 405 nm visible-light induced ligation of DASyd (**6a**)-BCN, an image containing spindle-shaped circles in Cy3 channel could be observed, colocalized with signal in the green channel (stained with membrane-embedding dye, DIO) on the cell surface after irradiation for 30 s. Meanwhile, almost no Cy3 signal can be seen for unexposed reaction for 30 min. Control group using unmodified Cetuximab also barely showed any signal after photo-stimulation, suggesting the excellent selectivity (Fig. 4b). Time-tracing for the DASAC conjugation and controlled imaging shed light on background interference until 150 min incubation (Figs. S14-S16 ESI†). These results together demonstrated that the fast and successful labelling of living cells within 30 s can be achieved via ligation of **6a** with BCN reporter by photo-acceleration instead of the process in dark. Therefore, the photo-induced DASyd-BCN ligation could be utilized as bioorthognal labelling tool.

Conclusively, via screening a library of DASyds, we were able to fully demonstrate the fast photo-responsive "click" reaction, realizing the covalent-bond ligation within 3 mins under 405 nm light exposure with only highly ring-strained alkynes. The

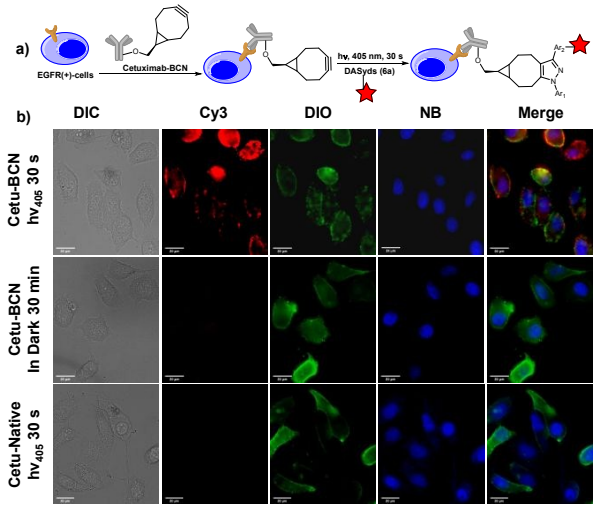


Fig. 4 Fluorescence microscopy imaging to characterize the localization of the DASyd **6a**-Alkyne photo-click labelling on living A549 cells. a) Schematic illustration of the procedure; b) Colocalization of photo-click labelling of Cetu-BCN on the cell surface (Cy3) and 3,3'-Dioctadecyloxacarbocyanine (DIO) versus DASAC reaction and background control, Scale bar: 20 µm, Exposure time: 2 s; NB = NucBlue™ Probe.

kinetic and protein photo-conjugation study provided strong evidence for us to distinguish the distinct photo-pathway from the background DASAC-reaction. Bifunctional DASyd **6a** was successfully applied to live cell surface labelling study with high fidelity. Therefore, the DASyd-BCN cycloaddition has emerged as a promising photo-click tool for detecting biomacromolecules in living system. To avoid the irreversible damage of high-energy photon to live cells, the introduction of Si or S atom on sydnone core might be one way to elongate the wavelength of photoexcitation.

Financial support was provided by the National Natural Science Foundation of China (21502130), the "1000-Youth Talents Program", and the Fundamental Research Funds for the Central Universities.

Conflicts of interest

The authors declare no competing financial interests.

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