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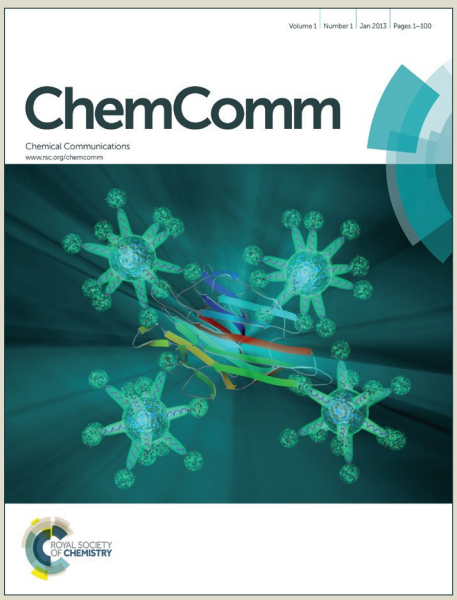

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Journal Name

COMMUNICATION

A Trifunctional Cyclooctyne for Modifying Azide-Labeled Biomolecules with Photocrosslinking and Affinity Tags

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A bicyclo[6.1.0]nonyne (BCN)-based cyclooctyne reagent bearing a photocrosslinking diazirine (DAz) group and a biotin affinity handle, BCN-DAz-Biotin, is reported. BCN-DAz-Biotin is capable of simultaneously delivering photocrosslinking and affinity tags to azide-labeled biomolecules, enabling photoactivated capture and enrichment/detection of interacting species in native contexts.

Bioorthogonal chemistry has revolutionized the investigation of biomolecules in living systems, proving to be particularly powerful for studying cellular components that are not genetically encoded, such as glycans and lipids.[1] The bioorthogonal chemical reporter strategy involves tagging a biomolecule of interest with a reactive handle, which can undergo a selective bioorthogonal reaction to deliver functional cargo to the biomolecule, thus enabling its analysis in a native context.[1, 2] The most commonly used chemical reporter is the azide, which is small in size, inert to the biological milieu, and capable of undergoing various bioorthogonal reactions, including Cu-catalyzed and strain-promoted azide–alkyne cycloadditions (CuAAC and SPAAC).[3-5] Strategies have been developed for incorporating azides into numerous types of biomolecules—including glycans, lipids, proteins, and nucleic acids—in various systems.[2] The type of cargo delivered to azide-labeled biomolecules via bioorthogonal reaction depends on the application, but typically include fluorescent tags to enable detection and affinity handles to enable enrichment.

The incorporation of photocrosslinking groups into biomolecules is a powerful approach to identifying their interactions in live cells, particularly when binding events are weak or transient.[6] For example, the Kohler lab has developed unnatural monosaccharides bearing small, photoactivatable diazirine groups that can be metabolically incorporated into glycoproteins and used to identify glycosylation-dependent protein–protein interactions.[7] Similar approaches have been developed to study various other types of biomolecular interactions.[8] Although diazirines are capable of being introduced into numerous biomolecules,[8] the development of new biomolecular labeling strategies and reagents is difficult and sometimes unsuccessful. As well, other photocrosslinking groups, such as aryl azides and benzophenones, are less amenable to metabolic incorporation due to their increased size.

In some cases, it may be preferable or necessary to capitalize on established chemical reporter strategies to first tag the biomolecule of interest with an azide, then deliver a diazirine group (or other photocrosslinker) to the biomolecule using an azide-specific reagent (Figure 1A). Recently, the Jewett group reported the first such reagent, an elegantly designed phosphine-based reagent called PhosDAz (**1**, Figure 1B) that delivers diazirines to azides via a "traceless" Staudinger ligation,[9] which was inspired by chemistry initially developed in the Bertozzi and Raines labs.[10-12] PhosDAz is readily synthesized from commercial materials and it selectively converts azides into amide-linked diazirines without adding significant bulk to the biomolecule, making it attractive for photocrosslinking applications. A disadvantage of PhosDAz for some applications is that it relies on relatively slow Staudinger ligation chemistry (often requiring multi-hour incubations), which limits the study of fast processes. For instance, our lab studies mycobacterial species, some of which divide every 2 hours. In addition, PhosDAz does not have a secondary handle for detection or enrichment of crosslinked species, which limits its use in complex settings and when relevant detection reagents (e.g., antibodies) are unavailable.

Here, we report a trifunctional cyclooctyne reagent, BCN-DAz-Biotin (**2**, Figure 1B), which is designed to deliver both diazirine and biotin tags to azide-labeled biomolecules via SPAAC, thus enabling covalent photocrosslinking of interactors and subsequent detection or enrichment of crosslinked species. BCN- DAz-Biotin is based on the azide-reactive cyclooctyne bicyclo[6.1.0]nonyne (BCN), which can be can be readily synthesized (or obtained commercially) and is among the faster cyclooctynes for SPAAC.[13, 14] The diazirine group was chosen as the photocrosslinker, and biotin was chosen as the affinity handle to allow either detection or enrichment using avidin products.

For the synthesis of BCN-DAz-Biotin, shown in Scheme 1, we used a modular strategy employing L-lysine as a trifunctional

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scaffold, similar to an approach previously used by Cravatt and co- workers to

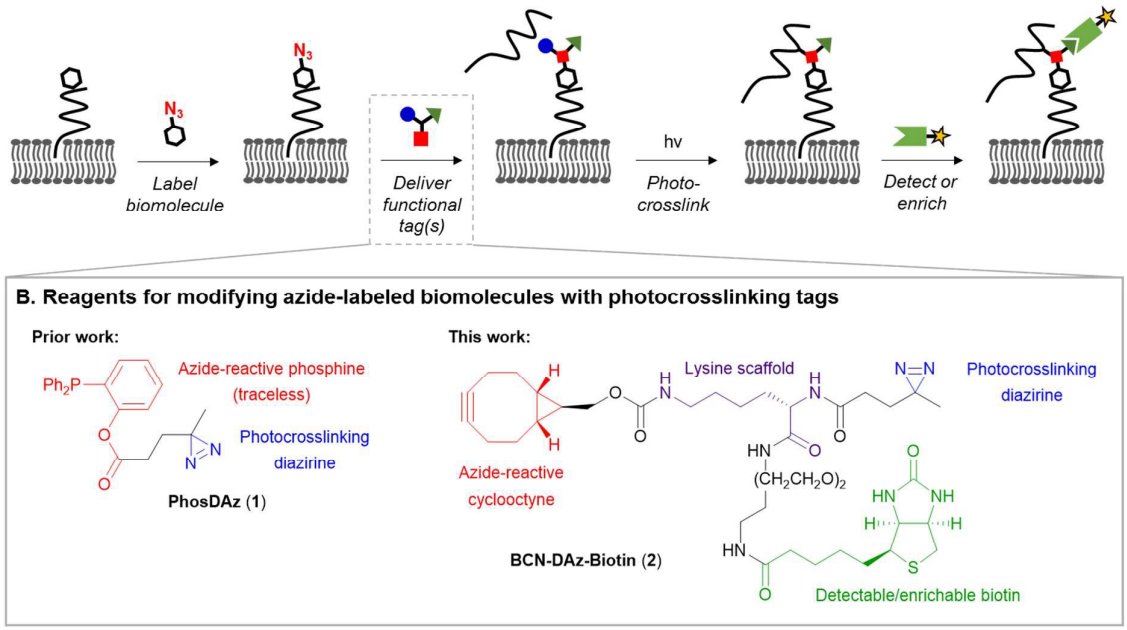

Figure 1. (A) Scheme for using bioorthogonal chemistry to probe biomolecular interactions. After labeling the biomolecule of interest using an established chemical reporter strategy, a bioorthogonal reagent can be used to modify the biomolecule with a photocrosslinking group and, in this work, an affinity tag. Interacting species can then be captured via photocrosslinking and subsequently enriched/detected. (B) Reagents for delivering photocrosslinking functionality to azide-labeled biomolecules, including the previously reported PhosDAz and the reagent reported herein, BCN-DAz-Biotin.

access other trifunctional reagents.[15] This strategy allows facile interchange of the type of cyclooctyne (or other bioorthogonal group), photocrosslinker, and detection tag, which provides flexibility to optimize all three components for specific applications. The synthesis began with mono-Boc-protected PEG diamine **3**,[16] which was sequentially biotinylated at the free amino group, Boc-deprotected with 50% trifluoroacetic acid (TFA), and linked to Boc-Lys(Fmoc)-OH (**4**). The resulting intermediate **5** was subjected to TFA-mediated Boc removal followed by coupling with diazirine-containing carboxylic acid **6**.[17] Removal of the Fmoc protecting group in **6** with 20% piperidine yielded compound **7**, which carried the biotin and diazirine groups, as well as a free amine to append the cyclooctyne. Finally, **7** was coupled with *exo*-BCN *para*-nitrophenyl carbonate (**8**)[13] to provide the target compound, BCN-DAz-Biotin (**2**).

With BCN-DAz-Biotin in hand, we evaluated its ability to react with azide-labeled biomolecules in living cells. Our group is focused on studying mycobacteria, including the pathogen *Mycobacterium tuberculosis*. Therefore, as a model system for studying the azide-specific reactivity of BCN-DAz-Biotin, we used an established strategy employing azido trehalose (TreAz) analogues to label trehalose glycolipids in live mycobacteria.[18, 19] First, *M. smegmatis* was treated with 6-TreAz, leading to efficient azide-labeling of glycolipids. The cells were then reacted with varying concentrations of BCN-DAz-Biotin (1–100 μM) for 30 minutes followed by incubation with avidin–488. Analysis of cellular fluorescence confirmed that BCN-DAz-Biotin was delivered to glycolipids in an azide- and concentration-dependent manner (Figure 2). The optimal concentration of BCN-DAz-Biotin was 50 μM, which provided a signal-to-noise ratio (S/N) of 12. Significant increases in fluorescence were also observed when using BCN-DAz-Biotin concentrations as low as 1 μM, allowing the reagent to be used sparingly if desired. If needed, enhanced sensitivity could be gained by exploiting the modular synthetic strategy to swap BCN for a more reactive cyclooctyne, such as BARAC.[20]

Having confirmed the azide reactivity and detectability of BCN-DAz-Biotin in a cellular context, we wished to demonstrate its third and key function, photocrosslinking. For this purpose, we adapted the model system developed by Jewett and co-workers, in which the reagent is tested for photocrosslinking with purified bovine serum albumin (BSA) in solution (Figure 3).[9] As a control, we also evaluated commercial DBCO-Biotin, which contains a cyclooctyne and biotin but lacks a photocrosslinker, and would therefore not be expected to bind to BSA in a UV light-dependent manner. First, BCN-DAz-Biotin (or DBCO-Biotin) was reacted with an azide-modified 488 fluorophore (Az488) to simulate ligation to a biomolecule while also providing a detection tag for subsequent analysis. The ligation

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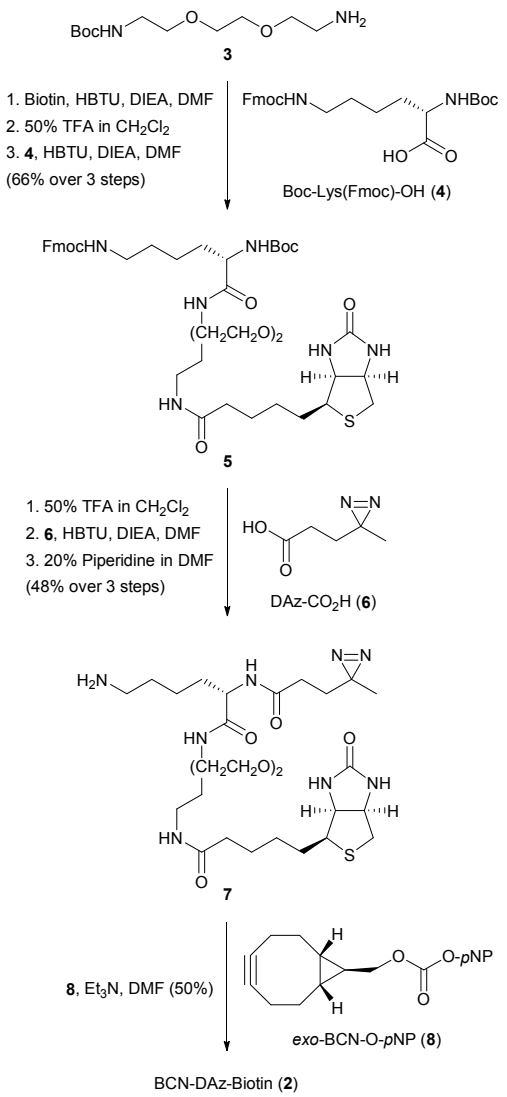

Scheme 1. Synthesis of BCN-DAz-Biotin. BCN, bicyclo[6.1.0]nonyne; Boc, *tert*-butoxycarbonyl; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; *p*NP, *para*-nitrophenyl; TFA, trifluoroacetic acid.

product was then incubated with BSA and irradiated at 365 nm for 20 min (or left non-irradiated). SDS-PAGE followed by in-gel fluorescence analysis confirmed that photocrosslinking occurred between BCN-DAz-Biotin and BSA only when exposed to UV light. Finally, to demonstrate that the photocrosslinked product could be detected via its biotin tag, Western blot analysis using avidin–HRP was performed. As expected, signal was only observed in the UV-irradiated BCN-DAz-Biotin sample. No photocrosslinking between the control compound DBCO-Biotin and BSA was observed by in-gel fluorescence or Western blot. Notably, we did not observe background signal in these experiments, which stood in contrast to the significant background observed when PhosDAz (**1**) was used in photocrosslinking experiments with BSA. Overall, the BCN-DAz-Biotin reagent functioned as designed in a model system, sequentially undergoing: (i) Efficient SPAAC reaction with an organic azide; (ii) photocrosslinking with a proximal biomolecule; and (iii)

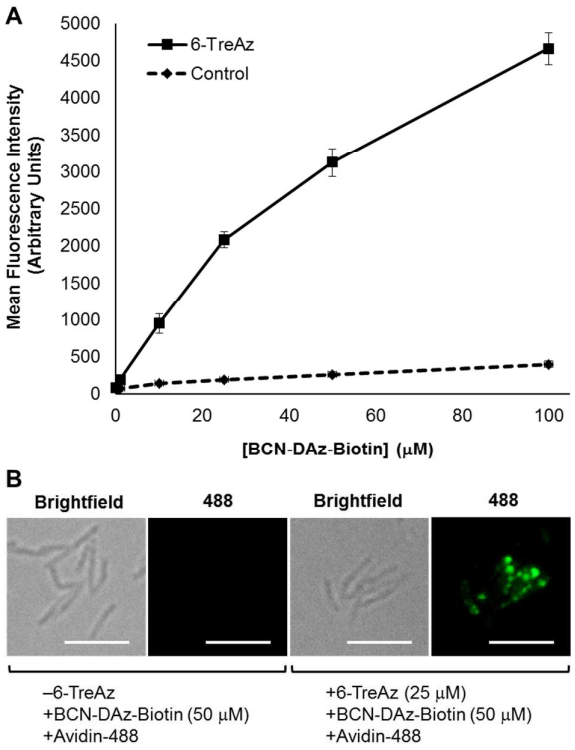

Figure 2. BCN-DAz-Biotin reacts with azide-labeled biomolecules in living cells. The bacterium *M. smegmatis* was cultured in the presence or absence of the chemical reporter 6-TreAz, then reacted with BCN-DAz-Biotin and stained with avidin-488. Flow cytometry (A) and fluorescence microscopy (B) analysis of azide-labeled cells reacted with BCN-DAz-Biotin at the indicated concentration and stained with avidin-488. Scale bars, 5 μm.

detection of the photocrosslinked product via its biotin handle. These experiments set the stage for performing in vivo crosslinking experiments with BCN-DAz-Biotin.

Conclusions

In summary, we developed the trifunctional cyclooctyne BCN-DAz-Biotin, which is the first reagent capable of modifying azide-labeled biomolecules with functional tags that allow both photocrosslinking and subsequent detection/enrichment of interacting species. We showed that BCN-DAz-Biotin, which was accessed via an efficient and modular synthetic strategy, possesses both of these functions by carrying out a proof-of-principle photocrosslinking experiment with BSA. Furthermore, the reagent successfully modified cell surface azides in the bacterium *M. smegmatis*, demonstrating its ability to be deployed in living systems. We envision that BCN-DAz-Biotin will facilitate the study of biomolecular interactions, particularly in cases where strategies already exist for incorporating azides into the biomolecule of interest. For instance, in relation to the cell labeling work presented herein, BCN-DAz-Biotin could be used to investigate host–pathogen interactions mediated by trehalose glycolipids during *M. tuberculosis* infection. Reagents such as PhosDAz and BCN-DAz-Biotin should be valuable tools given the large and growing number of strategies for incorporating azides into

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various types of biomolecules. PhosDAz and BCN-DAz-Biotin have complementary attributes

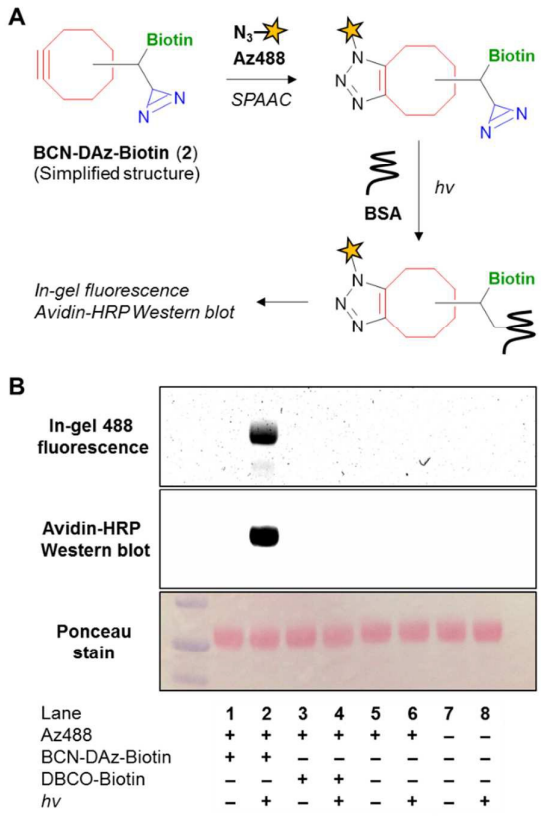

Figure 3. BCN-DAz-Biotin enables UV light-dependent photocrosslinking between an organic azide and a model protein, as well as subsequent detection of the photocrosslinked product. (A) Experimental workflow. BCN-DAz-Biotin (or control compound DBCO-Biotin) was reacted with an azide-modified 488 fluorophore (Az488), then incubated with BSA and irradiated with 365 nm UV light for 20 min (or left non-irradiated). (B) Reaction products were analyzed by SDS-PAGE with in-gel fluorescence to visualize 488-modified species (top), avidin-HRP Western blot to visualize biotinylated species (middle), and Ponceau stain to visualize protein loading (bottom). Protein ladder shows 37, 50, and 75 kDa markers.

that dictate their appropriateness for a given application: PhosDAz delivers diazirines to azides tracelessly and with minimal steric bulk, but does so with slower chemistry and lacks detection/enrichment capability; on the other hand, BCN-DAz-Biotin uses SPAAC to rapidly deliver diazirines to azides complete with detection/enrichment capability, but the size of the resulting conjugate is substantial. Future iterations of such reagents should combine fast and selective azide reactivity with the delivery of both photocrosslinking and affinity capabilities in the smallest form possible. Additionally, to expand the scope of this approach, similar reagents should be developed for other chemical reporters with desirable kinetics and selectivity, such as strained alkenes.[21] Work involving the development and applications of photocrosslinking reagents for mycobacteria research is ongoing in our lab.

Notes and references

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